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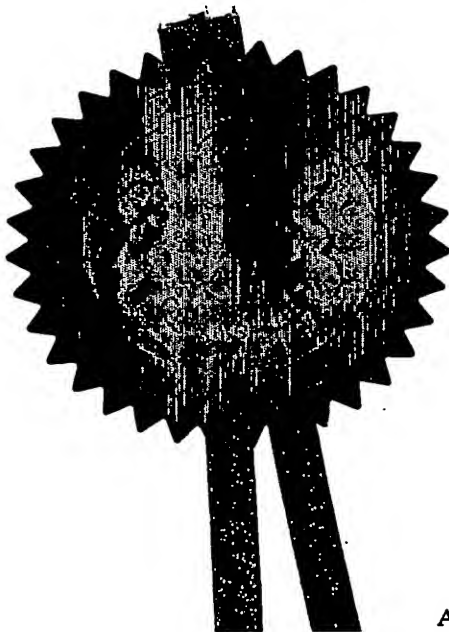
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1.	Your reference	GWS/PJG/24342

2.	Patent application number *(The Patent Office will fill in this part)*	**0228691.2**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Imperial College Innovations Limited
Imperial College
47 Prince's Gate
Exhibition Road
London SW7 2QA

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

7409 43 6003

4. Title of the invention

Bacterial Virulence Genes

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
United Kingdom

Patents ADP number *(if you know it)*

1081001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	Sequence listing (19 pages), Table (4 pages)

11.

I/We request the grant of a patent on the basis of this application.

Signature *Mathys & Squire*   Date

~~MATHYS & SQUIRE~~   ~~9 December 2002~~

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BACTERIAL VIRULENCE GENES

The present invention relates to the identification of genes involved in the virulence of *Actinobacillus pleuropneumoniae*, to the production of attenuated mutant strains

5 of *Actinobacillus pleuropneumoniae*, and to the identification of anti-bacterial agents which target the identified genes and their products.

A. pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious, respiratory disease of pigs. The disease is often fatal and as a result

10 leads to severe economic loss in the swine producing industry. An obligate parasite of the porcine respiratory tract, the incidence of infection of *A. pleuropneumoniae* has increased in recent years, notably where animals are subjected to intensive breeding conditions. Transmission of the bacterium is by aerosol, or by direct contact with infected pigs.

15

Pleuropneumonia can occur in swine of all ages, and there are no known associations with predisposing viral or bacterial infections. *A. pleuropneumoniae* infection may be chronic or acute, with the disease being characterised by increasingly severe pulmonary distress, which progresses rapidly to death, or to a

20 chronic infection resulting in a failure to thrive. A hemorrhagic, necrotic bronchopneumonia with accompanying fibrinous pleuritis is typically observed.

Fifteen different serotypes of *A. pleuropneumoniae* have been identified based on antigenic differences in their capsular polysaccharides, and their production of

25 extracellular toxins. Serotypes 1, 5 and 7 are the most common forms of *A. pleuropneumoniae* infection in the United States, whereas serotypes 1, 2, 5, 7 and 9 predominate in Europe.

Treatment of pigs infected with *A. pleuropneumoniae* usually involves the direct

30 injection of an antibiotic (typically tetracycline). Such a method is however labour

intensive, time consuming, and expensive, and can often be of limited use due to the rapid progression of the disease. Evidence from field and experimental studies has however shown that infection with *A. pleuropneumoniae* can provide a lifetime protection against subsequent reinfection, thereby suggesting that vaccination might

5   be a feasible alternative to antibiotic therapy. The development of an effective vaccine has so far been hampered by the antigenic diversity of the fifteen different serotypes.

In attempts to produce a vaccine composition, killed, whole cell bacteria were found

10   to provide only serotype-specific protection, but it has been shown that natural infection with a highly virulent serotype can stimulate a strong protective cross-immunity against multiple serotypes [Nielsen, Nord Vet Med. 31: 407-13 (1979); Nielsen, Nord Vet Med. 36: 221-234 (1984); Nielsen, Can J Vet Res. 29: 580-582 (1988); Nielsen, ACTA Vet Scand. 15: 80-89 (1994)]. Certain undefined live

15   attenuated mutants have also shown promise for cross- protection of swine [Inzana *et al*, Infect Immun. 61: 1682-6 (1993); Paltineanu *et al*, In International Pig Veterinary Society, 1992, p.214; Utrera *et al*, In International Pig Veterinary Society, 1992, p.213] but because of the uncertainties associated with vaccines comprising bacterial strains having such undefined spontaneous mutations, there exists a need

20   in the art for the rational construction of live attenuated strains which will safely stimulate protective immunity against homologous, and preferably heterologous *Actinobacillus pleuropneumoniae* serotypes. There also exists a need to identify the genes involved in *A. pleuropneumoniae* virulence, to facilitate the development of methods for identifying anti-bacterial agents which target those genes and/or their

25   products.

Published International patent application WO 00/61724 (Pharmacia & Upjohn) describes the use of signature tagged mutagenesis (STM) to identify genes responsible for the virulence of gram-negative bacteria. The development of

30   vaccines containing attenuated bacteria is also discussed. The work is also described in Fuller *et al*, Microbial Pathogenesis [2000] vol. 26; pp.39-51.

In the STM technique, a plurality of different bacterial strains, each of which carries a random mutation in its genome, is produced by means of transposon integration. The inserted transposons carry different DNA signature tags, which allows individual mutants to be differentiated from one other. The tags each comprise a variable

5   central region flanked by invariant "arms", the latter of which allow the variable central portion to be amplified by PCR. Multiple tagged mutant strains are collected in microtiter dishes, and then combined to form an "inoculum pool" for animal infection.

At an appropriate time after infection, bacteria are isolated from the infected animal,

10   and are combined to form a "recovered pool." The tags in the recovered pool and the tags in the inoculum pool are separately amplified, labelled, and used to probe filters arrayed with all the different tags from the mutants in the inoculum. Mutant strains with attenuated virulence are generally those which cannot be recovered from the infected animal, i.e. strains with tags that give hybridization signals when

15   probed with tags from the inoculum pool, but not when probed with tags from the recovered pool. The advantage of STM is that a large number of insertional mutant strains can be simultaneously screened in a single animal for loss of bacterial virulence. STM is more fully described in e.g. WO 96/17951.

20   After considerable research, the present inventors have provided improved methods and means for identifying genes involved in *A. pleuropneumoniae* virulence. This has led to the identification of a new family of "virulence genes" the members of which each comprise a nucleotide sequence which is selected from any one of those defined by SEQ ID NO: 1-56. As used herein, the term "virulence genes"

25   refers to genes of a bacterium whose correct function is involved in, e.g. required for the establishment and/or maintenance of an infection in a host animal. Virulence genes and their products, which may be polynucleotides (e.g. rRNA) and/or polypeptides, are thus involved in the pathogenesis of the bacterium, but might not be necessary for its growth *in vitro*.

30

Mutations in virulence genes can result in an "attenuated bacterium", i.e. one whose

ability to establish and/or maintain a bacterial infection in a host animal is reduced, as compared to a corresponding wild-type bacterium of the same strain and serotype. Attenuation of the bacterium may be confirmed by means of its administration to a subject animal, where its inability to cause a sustained infection

5   leading to e.g. chronic infection or death may be observed. The non-establishment of symptoms of porcine pleuropneumonia, e.g. of pulmonary distress, hemorrhagic necrotic bronchopneumonia and/or fibrinous pleuritis, may also be used as an index of bacterial attenuation. Attenuated bacteria are of commercial importance in vaccine compositions.

10

In one aspect, the present invention thus provides an attenuated *Actinobacillus pleuropneumoniae* bacterium.

In a particular embodiment, the present invention provides an attenuated

15   *Actinobacillus pleuropneumoniae* bacterium having a mutation in a gene which comprises a nucleotide sequence selected from the group consisting of SEQ ID NO.:1-56.

The mutation may inhibit or prevent the function of the gene, e.g. by reducing the

20   level of its expression (e.g. by reducing transcription and/or translation), and/or by impairing the biological activity of the gene product, e.g. by causing an inactive form of the gene product that is encoded by the mutated gene to be produced. The gene product may be a polynucleotide or polypeptide.

25   The mutation in the gene may be an insertion, deletion or substitution. It may be located in a region of the gene that encodes a polypeptide, or in a sequence responsible for, or involved in, the control of gene expression, e.g. in a promoter region. Mutations which involve a combination of one or more of insertion, deletion and/or substitution are also contemplated. Attenuated *A. pleuropneumoniae* strains

30   of the present invention likewise include those which have two or more mutations, each of which may be an insertion, a deletion, a substitution, or a combination

thereof. Multiple mutations may occur in a single gene, or they may occur in different genes within the same bacterial genome. They may result in an additive or synergistic level of attenuation. Multiple mutations may be prepared by design, or may fortuitously arise as a result of techniques for introducing a single mutation.

5

Deletion mutants include those in which all or part of the virulence gene is deleted. In one aspect, the mutation results in the deletion of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least

10 about 95%, at least about 98%, or at least about 99% of the virulence gene. A deletion may occur in the appropriate nucleotide sequence set forth in SEQ ID NO.:1-56, or it may occur in an adjacent region of the gene, e.g. in an associated control sequence.

15 Deletion mutants can be constructed using any technique which is well known to those skilled in the art. One strategy employs counter-selectable markers, and has been used to delete genes in many different bacteria. For details of this technique, reference may be made to e.g. Reyrat *et al*, Infection and Immunity [1998] vol. 66; pp.4011-4017, and Oswald *et al*, FEMS Microbiol Lett [1999] vol. 179; pp.153-160.

20

The process employs a double selection strategy, in which a plasmid is constructed which encodes both a selectable and a counter-selectable marker, with flanking DNA sequences being derived from both sides of the desired deletion site in the bacterial genome. The selectable marker is used to select for bacteria in which the

25 plasmid has integrated into the genome in the appropriate location and manner. The counter-selectable marker is then used to select for that small percentage of bacteria which spontaneously eliminate the integrated plasmid. A fraction of those resultant bacteria will contain the desired deletion, with no foreign DNA remaining.

30 In another technique for achieving a deletion, the *cre-lox* system is used for site specific recombination of DNA. The system consists of 34 base pair *lox* sequences

that are recognised by a bacterial *cre* recombinase. If the *lox* sites are introduced into the genomic DNA in an appropriate orientation, a sequence which is flanked by the *lox* sites can be excised by expression of the *cre* recombinase, one copy of the *lox* site being retained. Using standard recombination techniques, it is possible to

5 delete a sequence of interest in the *A. pleuropneumoniae* genome and to replace it with a selectable marker (e.g. a gene encoding kanamycin resistance) which is flanked by *lox* sites. Transient expression of the *cre* recombinase (e.g. from an electroporated suicide (non-replicating) plasmid containing the *cre* gene under the control of a promoter which functions in A. *pleuropneumoniae*) will then result in

10 efficient elimination of the *lox* flanked marker. This process results in a bacterial mutant containing the desired deletion, and one copy of the *lox* sequence.

In another approach to providing a deletion, a target sequence in the *A. pleuropneumoniae* genome can be deleted (replaced) with a marker gene, e.g. a

15 green fluorescent protein (GFP), β-galactosidase or luciferase. DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide vector for *A. pleuropneumoniae*. An expression cassette, containing a promoter active in *A. pleuropneumoniae* and operably linked to the appropriate marker gene, is then cloned between the flanking sequences. The plasmid is introduced into wild-type

20 bacteria and mutants which both incorporate and express the marker gene are isolated and examined for a desired recombination event (replacement of the wild-type gene with the marker gene).

As for insertion mutants, these include genes in which a stop codon is inserted into

25 an open reading frame (ORF) upstream (5') of the native, i.e. endogenous stop signal. A truncated, inactive form of the (polypeptide) virulence gene product may thus be expressed by the gene. Insertions which cause a frame-shift are likewise contemplated for the production of inactive products. Mutated virulence genes of *A. pleuropneumoniae* will also include those in which a transposon has been inserted

30 into a protein-coding or regulatory sequence of the gene. Attenuated bacteria produced by the STM technique are insertional mutants in which a virulence gene

has been rendered non-functional by insertion of a transposon sequence, e.g. Tn*10*.

Because insertional mutants, e.g. those generated by STM, will still contain all of the genetic information required for bacterial virulence, there is a risk that they could

5 revert to a pathogenic state, e.g. by spontaneous deletion of the inserted sequence. Accordingly, when preparing a vaccine or other therapeutic composition, based on an insertional mutant, e.g one identified by STM, it may be desirable to take the information obtained from that STM-derived strain, i.e. the identification of the disrupted virulence gene, and then recreate a mutant in which some, most, or all of

10 that virulence gene has instead been deleted. This should preclude the possibility that the bacterium will revert to a virulent state, thereby to cause pathology, rather than prophylactic protection of a host animal to which it is administered.

In a related aspect, the present invention provides a composition containing the

15 attenuated *A. pleuropneumoniae* bacterium according to the first aspect of the invention. The composition may be an immunogenic composition, i.e. one which is capable of eliciting an antibody response in a host animal to which it is administered. The composition may be a therapeutic (e.g. prophylactic) composition. It may be a vaccine, preferably one whose administration to an animal confers a degree of

20 protection against subsequent infection with serotypes and strains of *A. pleuropneumoniae* different from that/those which is/are present in the vaccine itself (cross-protection). The invention further provides for the use of an attenuated *A. pleuropneumoniae* bacterium of the first aspect of the invention for the manufacture of a medicament (e.g. vaccine) for preventing or alleviating an infection of an animal

25 with *A. pleuropneumoniae* and/or for preventing or alleviating symptoms associated with such infection, e.g. for the prophylactic protection of swine against porcine pleuropneumonia.

In order for an attenuated bacterium to be effective in a vaccine composition, the

30 (one or more) mutation(s) in the virulence gene(s) should be significant enough to prevent the pathogen from evoking severe clinical symptoms, but insignificant

enough to allow a limited replication and growth of the bacteria in the host animal, thereby to elicit appropriate defence mechanisms resulting in immunological memory. Mutations in genes which are required for (as distinct from merely involved in) virulence are preferred.

5

Compositions of the invention may contain a plurality of different attenuated *A. pleuropneumoniae* bacteria, e.g. bacteria having different mutations in the same virulence gene, and/or bacteria having similar, or different, mutations in two or more different genes. While it is possible for an avirulent (attenuated) bacterium of the
10 present invention to be administered alone, it is preferably administered in a solid or liquid composition containing one or more suitable adjuvants, diluents, carriers, vehicles and/or excipients, as discussed elsewhere herein. Compositions according to, and for use in the present invention, may thus comprise one or more of these substances, which should be pharmacologically "acceptable", in the sense of being
15 both compatible with the attenuated bacterium, and not deleterious to the animal to be immunized. Additional substances are preferably both sterile and pyrogen free.

Examples of adjuvants which may be used in the present invention include oil-based adjuvants such as Freund's Complete Adjuvant and Freund's Incomplete Adjuvant,
20 mycolate-based adjuvants (e.g. trehalose dimycolate), bacterial lipopolysaccharide (LPS), peptidoglycans (e.g. mureins, mucopeptides, or glycoproteins such as N-Opaca, muramyl dipeptide [MDP], or MDP analogs), proteoglycans (e.g. those extracted from *Klebsiella pneumoniae*), streptococcal preparations (e.g. OK432), BiostimTM (e.g. O1K2), the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039,
25 aluminum hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic polyols, the Ribi adjuvant system (see e.g. GB-A-2 189 141) and interleukins, particularly those that stimulate cell-mediated immunity. An adjuvant consisting of extracts of *Amycolata*, a genus of bacteria of the order Actinomycetales, has also been described (US 4,877,612).
30 Proprietary adjuvant mixtures are commercially available. The adjuvant to be used will depend, at least in part, on the recipient animal. The amount of adjuvant to be

administered will also depend on the type and size of the animal. Optimal dosages may be readily determined by routine methods well known to those skilled in the art.

5 As for any diluent(s), carrier(s), vehicle(s) and/or excipient(s), these may be liquid, semisolid, or solid in nature. Any suitable substance known to those skilled in the art may be used. Exemplary substances include polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

10

The compositions of the invention may be packaged in various forms which maybe convenient for, or adapted to, a particular route of delivery. Therapeutic compositions (e.g. vaccines) may be suitable for administration to a subject animal by for example intravenous, intradermal, intramuscular, intramammary, 15 intraperitoneal and/or subcutaneous injection, and/or by oral, sublingual, nasal, anal or vaginal delivery. The treatment may consist of a single dose, or of a plurality of doses over a period of time. Decisions on administration regimes are well within the ambit of those skilled in the art. The composition may be formulated and packaged as appropriate.

20

In a further aspect, the present invention provides an isolated polynucleotide encoding a gene product which is naturally involved in (e.g. required for) the virulence of A. pleuropneumoniae. The sequence of the polynucleotide is thus found in a virulence gene of A. pleuropneumoniae. The invention also provides for 25 a polynucleotide encoding a gene product which is not naturally found in A. pleuropneumoniae, but whose expression therein is capable of modulating (e.g. of decreasing) the virulence of that bacterium. Such a polynucleotide may be introduced into the bacterium to cause such expression (e.g. by transformation, transfection or electroporation). Expression may result in an attenuated strain of the 30 bacterium, e.g. for use in a vaccine or other immunogenic composition. Polynucleotides which are not naturally found in A. pleuropneumoniae but which are

capable of modulating the virulence of that bacterium by their direct interaction with A. *pleuropneumoniae* virulence genes or gene products (rather than by indirect action through their own gene products) are also contemplated.

5   Any gene product of a polynucleotide according to the present invention may be a polynucleotide (e.g. RNA) or a polypeptide. Polypeptide gene products are herein referred to as "virulence polypeptides", and are involved in/capable of modulating the virulence of A. *pleuropneumoniae*, whether or not they are naturally encoded by the wild-type bacterial genome. Polynucleotide gene products which can affect the

10   virulence of A. *pleuropneumoniae* include e.g. antisense RNA and ribozymes. An effect on bacterial virulence may be demonstrated for any gene product by determining the extent to which a strain of the bacterium A. *pleuropneumoniae* which expresses the product can establish and/or maintain an infection in an animal as compared with A. *pleuropneumoniae* of the same strain and serotype which does

15   not express the product.

According to this aspect, the present invention more particularly provides for an isolated polynucleotide comprising (e.g. consisting of): (a) a nucleotide sequence selected from the group consisting of SEQ ID NO.: 1-56 (such sequences are found

20   within the virulence genes of A. *pleuropneumoniae* as identified elsewhere herein); (b) a nucleotide sequence encoding the polypeptide which is encoded by the nucleotide sequence recited in (a); (c) a nucleotide sequence which hybridizes to the nucleotide sequence of (a) and/or (b), or to its complement, under conditions of moderate to high stringency; or (d) a fragment of any one of the nucleotide

25   sequences of (a)-(c), which fragment retains an immunological property and/or a biological activity of the recited nucleotide sequence of (a)-(c). Polynucleotides of the present invention may be DNA or RNA, as described elsewhere herein.

Nucleotide sequences of type (b) can be readily identified once an amino acid

30   sequence is derived from the nucleotide sequence of (a), and account is taken of the genetic code.

As to (c), exemplary conditions of moderate stringency include a final wash in a buffer comprising 2xSSC/0.1% SDS, at 35°C to 45°C. Exemplary conditions of high stringency include a final wash in a buffer comprising 0.2xSSC/0.1 % SDS, at 65°C to 75°C. It is understood in the art that conditions of equivalent stringency can be

5   achieved by means of variation of temperature and buffer or of salt concentration. In this regard, reference may be made to Ausubel *et al*, Protocols in Molecular Biology, John Wiley & Sons (1994), pp.6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on length and percentage of guanosine/cytosine (GC) base pairing. Hybridization conditions

10   can be calculated as described in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

For fragments of type (d), knowledge of the nucleotide sequence according to (a),

15   (b) or (c) makes available to the skilled person every possible fragment of that nucleotide sequence. Fragments may be at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50 nucleotides in length. They may be capable of hybridising to the nucleotide sequence of (a), (b) or (c), or to its complement, under conditions of moderate to high stringency, optionally

20   in the presence of competitive nucleic acid, e.g. in the presence of nucleic acid from a cDNA or mRNA library derived from *A. pleuropneumoniae*. The retained immunological property may be an ability of the fragment to be bound specifically by an antibody which binds specifically to the full length nucleotide sequence. Binding affinities of at least 10^7 M^{-1} are contemplated. A retained biological activity

25   may be an ability of the fragment to encode a polypeptide which shares a binding capability with the polypeptide encoded by the full length nucleotide sequence, e.g an ability to bind to one of the ligands to which it naturally binds *in vivo*, or the same antibody.

30   As will be readily apparent to the person of skill in the art, the identification of virulence genes of *Actinobacillus pleuropneumoniae* enables the identification of

corresponding (i.e. homologous) genes in other related bacteria, e.g. in other members of the *Pasteurellaceae* family. In isolated form, such gene sequences may be encompassed within the claimed polynucleotides comprising (or consisting of) a nucleotide sequence according to type (c) above. Southern hybridization using the

5   nucleotide sequences of type (a) (or fragments thereof) as probes, can identify such related sequences in genomic, cDNA or mRNA libraries from other bacteria. Alternatively, the use of PCR, with primers comprising the *A. pleuropneumoniae* sequences or fragments thereof, can be equally as effective for identifying virulence genes across species boundaries. As a further alternative (which does not rely on

10 hybridisation but still identifies homologous genes) complementation of an *A. pleuropneumoniae* mutant strain with a polynucleotide (e.g. DNA or mRNA) library from another species, can be used to identify genes having the same, or a related biological activity. In short, polynucleotides of the invention, which comprise a nucleotide sequence according to type (c), may be found in, or constitute, virulence

15 genes of other bacteria related to *A. pleuropneumoniae*.

Identification of related virulence genes can lead to the production of an attenuated strain of the other organism by mutation in that gene, and may therefore be used in the production of vaccines and other immunological compositions. In yet further

20 aspects, the present invention thus provides: (1) an attenuated bacterium (e.g. of the *Pasteurellaceae* family) containing a mutation in a gene comprising a nucleotide sequence which is capable of hybridising to any one of the nucleotide sequences defined by SEQ ID NO:1-56, under conditions of moderate to high stringency; (2) a composition (e.g. a vaccine) containing such a bacterium; and (3) the use of such

25 a bacterium for the manufacture of a medicament (e.g. a vaccine) for the therapeutic treatment or prophylactic protection of an animal against infection by the corresponding wild-type bacterium (or a different strain or serotype thereof).

As explained elsewhere herein, polynucleotides of the present invention of types (a)-

30 (d) may be e.g. DNA, RNA, or peptide nucleic acids, as described for example in Corey, TIBTECH 15: 224-229 (1997). DNA sequences include those derived

(extracted or amplified) from genomic DNA, or from cDNA, as well as wholly or partially chemically synthesized nucleotide sequences. Amplification may be effected by any method well known to those skilled in the art, e.g. by PCR.

5 "Chemically synthesized" as used herein refers to purely chemical, as opposed to enzymatic (e.g PCR-based) methods for producing polynucleotides. "Wholly" synthesized polynucleotides are those produced entirely by chemical means, and "partially" synthesized polynucleotides include those wherein only portions of the resulting sequence are produced by chemical means.

10

The polynucleotides of the invention, or their gene products, may be ribozymes. Such polynucleotides may comprise or consist of an (RNA) nucleotide sequence according to type (c) above, i.e. one which is capable of hybridising to any of the nucleotide sequences recited in (a) or (b) under conditions of moderate to high

15 stringency. For a review of ribozyme technology, reference is made to Gibson and Shillitoe, Mol. Biotech. 7: 125137 (1997). A ribozyme can be utilized to inhibit the translation of mRNA (e.g. mRNA transcribed from virulence genes) in a sequence specific manner, e.g. by: (i) the hybridization of a complementary RNA sequence in the ribozyme to the target mRNA; and (ii) cleavage of the hybridized target mRNA

20 through nuclease activity inherent to the ribozyme. The polynucleotides of the invention may therefore be used to inhibit the expression of virulence genes of *A. pleuropneumoniae* or of other related e.g. *Pasteurellaceae* bacteria. Ribozymes can be identified by empirical methods, but they may be specifically designed based on accessible sites on the target mRNA, e.g. on the mRNA transcription product of the

25 nucleotide sequences defined by SEQ ID NO:1-56 (see also Bramlage *et al*, Trends in Biotech 16: 434-438 (1998)).

Delivery of ribozymes to target cells, in order to control mRNA translation, may be accomplished using delivery techniques well known to those skilled in the art.

30 Exogenous delivery methods can include the use of targeting liposomes or direct local injection. Endogenous methods include the use of viral vectors and of non-viral

plasmids.

Certain polynucleotides of the present invention may also be used to modulate the transcription of virulence genes in *A. pleuropneumoniae*, e.g. by means of

5 oligonucleotide-directed triplet helix formation. For a review of this technique, reference is made to Lavrovsky *et al*, Biochem. Mol. Med. 62: 11-22 (1997). Triplet helix formation is accomplished using sequence specific oligonucleotides which hybridize to double stranded DNA in the major groove (as defined in the Watson-Crick model). Hybridization of such sequence specific oligonucleotides can

10 thereafter modulate the activity of DNA-binding proteins, including, for example, transcription factors and polymerases. Preferred target sequences for hybridization include transcriptional regulatory regions that modulate the expression of virulence genes. Oligonucleotides which are capable of triplet helix formation are also useful for site-specific covalent modification of target DNA sequences in virulence genes.

15 Oligonucleotides useful for covalent modification are coupled to DNA damaging agents as described for example in Lavrovsky, *et al*, Biochem. Mol. Med. 62: 11-22 (1997).

In yet another aspect, the present invention provides a vector comprising a

20 polynucleotide according to the present invention. The vector may be DNA or RNA in nature. It may contain one or more control sequences operably linked to the polynucleotide, in order to promote its transcription, and optionally enable its translation, in a suitable host cell under appropriate conditions. Vectors may be plasmids, or viral vectors. They may be capable of autonomous replication. Control

25 sequences may be endogenous (i.e. found within the wild-type bacterium from which the polynucleotide of the invention is derived, e.g. *A. pleuropneumoniae*) or they may be exogenous (e.g. a control sequence found in a related bacterium, e.g in another member of the *Pasteurellaceae* family). Control sequences include *inter alia* promoters and transcription terminators.

30

Host cells containing (e.g. transformed, transfected, or electroporated with) a

polynucleotide or vector of the present invention are further contemplated. Such host cells may be prokaryotic or eukaryotic in nature, either stably or transiently transformed, transfected, or electroporated with a polynucleotide or vector of the present invention, in a manner which permits the transcription of the polynucleotide,

5 and optionally enables translation of the resultant mRNA. Host cells of the invention include bacterial, yeast, fungal, invertebrate, and mammalian cells. Viruses containing a polynucleotide or vector of the present invention are also contemplated.

Host cells of the present invention may be useful in methods for the large scale

10 production of virulence polypeptides (where the polynucleotide of the invention encodes a polypeptide sequence). The cells are grown in a suitable culture medium under favourable conditions, and the desired polypeptides are isolated from the cells, or from the medium in which the cells are grown, by any purification technique well known to those skilled in the art, e.g. by capture on an affinity column. Such

15 cells may be a valuable source of immunogen for the development of antibodies which specifically bind to the virulence polypeptide. Any suitable host cell may be used for the expression of a polynucleotide according to the present invention.

For this and other purposes, polynucleotides of the present invention may be

20 manipulated using standard techniques well within the ambit of those skilled in the art, e.g. the techniques described in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989). By way of example, the polynucleotides of the invention may be amplified and cloned by e.g. PCR, e.g. using genomic DNA, cDNA or mRNA as a

25 template. For ease of inserting the cloned sequence into vectors; e.g. expression vectors, PCR primers may be chosen so that the PCR-amplified sequence has a restriction enzyme site at the 5' end which precedes the initiation codon ATG, and a restriction enzyme site at the 3' end after the termination codon TAG, TGA, or TAA. If desirable, the codons in the cloned sequence may be changed, without

30 changing the amino acids, according to the codon preference of an intended host cell, e.g. *E. coli*, as described by Grosjean and Fiers, Guide, 18: 199-209 (1982) and

Konigsberg and Godson, Proc. Natl. Acad. Sci. (USA), 80: 687-691 (1983). Optimization of codon usage may lead to an increase in the expression of the gene product when produced in that host.

5   If a virulence polypeptide is to be produced extracellularly, e.g. in the periplasm of a host bacterium, or in the culture medium surrounding a host cell, then the polynucleotide of the present invention may be cloned without its initiation codon, and placed into an expression vector behind an appropriate signal sequence. A number of signal sequences from both prokaryotes and eukaryotes are known to

10 function in the secretion of recombinant proteins. During the protein secretion process, the signal peptide may be removed by a signal peptidase to yield the mature protein.

To simplify the purification of virulence polypeptides, a purification tag may be added

15 to the 5' and/or 3' end of the polypeptide encoding sequence. Commonly used purification tags include a stretch of six histidine residues (US Patent Nos. 5,284,933 and 5,310,663), a streptavidin-affinity tag described by Schmidt and Skerra, Protein Engineering, 6: 109-122 (1993), a FLAG peptide (Hopp et al., Biotechnology, 6: 12051210 (1988)), glutathione S-transferase (Smith and Johnson,

20 Gene, 67: 31-40 (1988)), and thioredoxin (LaVallie et al., BiolTechnology, 11: 187-193 (1993)). To remove these tags, a proteolytic cleavage recognition site may be inserted at the fusion junction. Commonly used proteases are factor Xa, thrombin, and enterokinase.

25 In a further aspect, the present invention provides a method of producing a virulence polypeptide encoded by a polynucleotide of the invention. The method may comprise the steps of: (i) culturing a host cell according to the present invention under conditions which permit the expression of the polypeptide; and (ii) recovering and optionally isolating the expressed polypeptide from the host cell, or from its

30 surrounding medium. Identification of the polynucleotides of the present invention readily makes available their encoded polypeptides, so such polypeptides can

alternatively be chemically synthesised.

Polypeptides encoded by polynucleotides of the present invention (and variants of such polypeptides) are a further aspect of the invention. Such polypeptides are

5 "virulence polypeptides", as discussed elsewhere herein. Compositions containing such polypeptides are further contemplated, and these include vaccines and other immunogenic compositions. Such compositions may contain one or more adjuvants, carriers, diluents, vehicles or excipients, as discussed elsewhere herein.

10 Variants include biologically and/or immunologically active fragments of the polypeptide encoded by a polynucleotide of the invention, e.g. fragments of the polypeptide encoded by the polynucleotide sequence defined in any one of SEQ ID NO.:1-56, fusions of the polypeptides or fragments thereof with e.g. heterologous polypeptides (thereby to produce chimeras), and analogs of the polypeptides, e.g.

15 polypeptides in which one or more amino acids has been deleted, replaced or added: (i) without loss of one or more of the biological activities and/or immunological characteristics specific for the polypeptide; or (ii) with specific disablement of a particular biological activity of the polypeptide. Replacement may occur using conservative amino acids.

20

Virulence polypeptides having an additional methionine residue at position -1 are contemplated, as are virulence polypeptides having additional methionine and lysine residues at positions -2 and -1. Variants of these types are particularly useful for recombinant protein production in bacterial cell types. The invention also embraces

25 variants having additional amino acid residues which result from the use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as a fusion protein with glutathione-S-transferase (GST) provide the desired polypeptide having an additional glycine residue at position -1 after cleavage of the GST component from the desired polypeptide.

30 Variants of polypeptides of the present invention also include those in which a leader or signal sequence has been removed, e.g. a mature form.

Variant polypeptides include those which have at least about 99%, at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, and at least about 50% sequence identity and/or homology with a virulence

5 polypeptide encoded by a nucleotide sequence as defined in any one of SEQ ID NO.:1-56. Percentage "identity" of an amino acid sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the virulence polypeptide, after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity,

10 and not considering any conservative substitutions as part of the sequence identity. Percent "homology" of an amino acid sequence with respect to a virulence polypeptide encoded by any one of SEQ ID NO:1-56 refers to the percentage of amino acid residues in the candidate sequence that are identical with the residues in the virulence polypeptide after aligning the two sequences and introducing gaps,

15 if necessary, to achieve the maximum percent sequence identity, and also considering any conservative substitutions as part of the sequence identity.

By conservative substitution is meant exchange of one amino acid for an amino acid of the same type, the types being as follows:

20

Aliphatic Non-polar :	G A P I L V
Polar-uncharged sulphur : C M	
Polar-uncharged hydroxyl:	S T
Polar-charged acidic:	D E N Q
Polar-charged basic:	K R H
Aromatic:	F W Y

25 (Polar-charged basic:) (K R H)

In a still further aspect, the present invention extends to antibodies which specifically recognize the polynucleotides or polypeptides of the present invention. Antibodies

30 may be monoclonal or polyclonal antibodies. They include chimeric antibodies, e.g. humanized antibodies, e.g. complementary determining region (CDR)-grafted

antibodies, as well as fragments of antibodies (which may be chimeric) e.g. Fab, F(ab')$_2$, Fd and single chain antibodies. They also include compounds having CDR sequences which specifically recognize a polynucleotide or polypeptide of the present invention. The invention also provides anti-idiotype antibodies which

5   specifically bind to antibodies of the present invention.

The term "specific for" indicates that the variable regions of the antibodies of the invention exclusively recognize a target polypeptide or polynucleotide (i.e. they are able to distinguish a single polypeptide or polynucleotide from related polypeptides

10   or polynucleotides). Binding affinities of at least about 10^7 M^{-1}, at least about 10^8 M^{-1}, or at least about 10^9 M^{-1} are contemplated. That is not to say that the antibodies will not also interact with other proteins (for example, S. aureus protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the

15   molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practised by those skilled in the art. For a comprehensive discussion of such assays, reference may be made to Harlow et al. (Eds), Antibodies. A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY (1988), Chapter 6.

20

According to a still further aspect of the present invention, materials and methods are provided for identifying an anti-bacterial agent which is capable of modulating the function of an A. pleuropneumoniae virulence gene identified by the present invention, or of a homologous gene in a related species. Agents which are capable

25   of modulating the function of such virulence genes are likewise contemplated per se. More particularly, methods of the present invention include screening potential agents for their ability to interfere with the expression and/or biological activity in a host bacterium of the gene products encoded by the nucleotide sequences set forth in any one of SEQ ID NO:.1-56. Agents that interfere with the expression of

30   virulence gene products include (but are not limited to) anti-sense polynucleotides and ribozymes containing nucleotide sequences which are homologous to any one

of the nucleotide sequences of SEQ ID NO:.1-56 (such that they bind to its complement). The invention further embraces methods of modulating the transcription of such virulence genes through the use of oligonucleotide-directed triplet helix formation.

5

Compositions containing an anti-bacterial agent identified in accordance with the present invention are a still further aspect of the present invention, in addition to a method of treating an animal suffering from a *Pasteurellaceae* (e.g. an *A. pleuropneumoniae*) infection by administration of such an anti-bacterial agent or

10 composition. The invention also embraces the use of such an agent or composition for the manufacture of a medicament for the treatment of a *Pasteurellaceae* infection, e.g. an *A. pleuropneumoniae* infection and/or porcine pleuropneumonia.

Agents that interfere with the function of virulence genes encompass: (i) non-

15 functional variants of the virulence gene products which may be capable of out-competing the wild-type product for its physiological binding sites; and (ii) binding partners of the virulence gene products, which sequester and optionally destroy said products. Agents also include allosteric enzyme inhibitors, in the case where the virulence gene product is an enzyme.

20

Inhibitory agents may be identified by means of high throughput screening assays using the polynucleotides of the present invention or their encoded products (which may be RNA polynucleotides or polypeptides). Where the screen employs polypeptide gene products having enzymatic activity, assays are established which

25 are based on that activity, and potential agents screened for an ability to modulate (e.g. inhibit) the activity. For virulence gene products which exert their function by interacting with a polypeptide or nucleic acid, binding assays are established to measure such interaction, and potential agents are screened for an ability to modulate, e.g. to inhibit the interaction.

30

In one example of a binding assay, the virulence gene product is immobilized on a

solid support, and an interaction with a (physiological) binding partner is assessed in the presence and absence of a putative inhibitor compound. In another example, interaction between the virulence gene product and its binding partner is assessed in a solution assay, both in the presence and absence of a putative inhibitor

5 compound. In both assays, an inhibitor is identified as a compound that decreases binding between the virulence gene product and its binding partner. Assays for use when the virulence gene product and its binding partner are both polypeptides include variations of the di-hybrid assay, wherein an inhibitor of protein/protein interaction is identified by detection of a positive signal in a transformed or

10 transfected host cell, as described for example in published International patent application WO 95/20652.

Candidate inhibitors contemplated for use in the methods of the present invention include compounds selected from libraries of potential inhibitors. A number of

15 different libraries can be used as a source of small molecule modulators, including: (1) chemical libraries; (2) natural product libraries; and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules.

Chemical libraries consist of structural analogs of known compounds. Natural

20 product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (i) fermentation and extraction of broths from soil, plant or marine microorganisms; or (ii) extraction of plants or marine organisms. Natural product libraries are further discussed in e.g. Science 282: 63-68 (1998). As for combinatorial libraries, these are composed of

25 large numbers of peptides, oligonucleotides, or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. For a review of combinatorial chemistry libraries, reference may be made to Myers, Curr. Opin. Biotechnol. 8: 701-707 (1997).

30

Still other candidate inhibitors, which are contemplated for use in the methods of the

invention, may be specifically designed. These include soluble forms of the binding partners of virulence polypeptides. The term "binding partners" as used herein encompasses antibodies (although in certain embodiments these are excluded) including fragments and derivatives thereof, and modified compounds comprising

5   antibody domains that specifically bind to the target gene product.

When a polypeptide binding partner for a virulence gene product is not known, assays that identify physiological, or non-physiological and hence sequestering binding partners may be employed, e.g. assays using affinity columns on which the

10   virulence gene product is immobilised. Alternatively, binding interactions between polypeptides may be identified using the yeast two-hybrid system as described in Fields and Song, Nature, 340: 245-246 (1989), and Fields and Sternglanz, Trends in Genetics, 10: 286-292 (1994). Other assays which may be used to search for agents that bind to a polypeptide virulence gene product include that described in

15   US Patent No. 5,585,277, which relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a target virulence polypeptide, the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence

20   of a test ligand which binds the target protein, than in the absence of a ligand. Binding of the ligand to the target protein can be determined by any method which distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand,

25   including, but not limited to metals, polypeptides, including proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

Another method for identifying ligands of a virulence gene polypeptide is that described in Wieboldt et al, Anal. Chem., 69: 1683-1691 (1997). That technique

30   screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target virulence polypeptide. Agents that bind to the target are

separated from other smaller library components by centrifugal ultra-filtration. The specifically selected molecules that are retained on the filter are subsequently liberated from the target protein and analysed, e.g. by HPLC and pneumatically assisted electrospray (ion spray) ionization MS. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for libraries of small molecules.

Binding agents identified by the initial screens maybe evaluated for their effect on virulence *in vivo*. Agents that interfere with bacterial virulence can prevent the establishment of an infection, or reverse the outcome of an infection once it is established. Binding agents may be included in compositions containing pharmaceutically acceptable adjuvants, excipients, diluents, carriers or vehicles, as described elsewhere herein.

Numerous additional aspects and advantages of the invention will now become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently prepared embodiments thereof.

EXPERIMENT 1

A.   Materials and Methods

1.   *Bacterial strains and growth conditions*

A spontaneously nalidixic acid-resistant derivative of the virulent *Actinobacillus pleuropneumoniae* serotype 1 strain 4074 was selected using conventional methods and designated 4074 Nal^R. The virulence of this strain was verified by passage in pigs. *A. pleuropneumoniae* strains were propagated at 37°C with 5% CO_2 on brain-

heart infusion (BHI) plates supplemented with 10% Levinthal's base (BHI$_L$), or at 37°C in Colombia broth with 5μg/ml β-nicotinamide adenine dinucleotide (NAD) and 11mM CaCl$_2$.

5   The *E. coli* strains used in this study were: S17 λ pir [*recA thi pro hsdR$^-$M$^+$ RP4::2-Tc::Mu::Km* Tn7 lysogenized with λ pir phage] as described in Miller and Mekalanos (1988) J. Bacteriol. 170, pp.2575-2583; CC118 λ pir [Δ*(are-leu) araD* Δ *lacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1* lysogenized with λ pir phage] as described in de Lorenzo *et al*, (1990) J. Bacteriol. 172, pp. 6657-6667; and XL1-Blue (*recA1*

10   *endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI^q* ZΔM15 Tn*10* (Tet^r)]). *E. coli* strains were maintained in Luria-Bertani (LB) medium with appropriate antibiotics used at the following concentrations: ampicillin 100μg/ml; kanamycin 100μg/ml; nalidixic acid 20μg/ml; tetracycline 10μg/ml.

15 2.   *DNA manipulations, amplification, labelling, and hybridisation of tags*

Chromosomal DNA was isolated from *A. pleuropneumoniae* using a QIAamp mini DNA kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was isolated from *E. coli* using a Qiagen plasmid midi kit, again according to the

20   manufacturer's instructions. DNA manipulation was performed according to standard techniques as described in Sambrook *et al*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989). Probes for southern blots were labelled with [digoxigenin (DIG)-11]-dUTP using a PCR DIG Probe Synthesis Kit (Roche) as described by the manufacturer.

25   Amplification and labelling of DNA signature-tags was performed as described in Hensel *et al* (1995) Science 269 pp.400-403. Tags were initially amplified from genomic DNA by means of PCR using the following primers:

P6 (5'-CGACTACAACCTCAAGCT-3')

30   P7 (5'-CGACCATTCTAACCAAGC-3')

The amplified tags were purified on a 2% Nusieve GTG agarose gel and radiolabelled with [^{32}P]-dCTP in a second PCR, again using P6 and P7. The cycling conditions for both PCRs were as follows: denaturation and enzyme activation 12min at 95°C, followed by 20 cycles of: 95°C for 30s, 50°C for 45s and 72°C for

5 10s. Reactions were performed using 1 unit of AmpliTaq Gold (Perkin-Elmer) polymerase in a Perkin-Elmer 2400 thermocycler. Prior to use, the invariant flanking DNA was separated from the variable signature-tag by digestion with *HindIII* for 18h at 37°C.

10 For colony blots, *E. coli* S17 λ pir. strains harbouring each of the pLOF/TAG 1-48 plasmids were grown to mid-exponential phase and 100 μl of each culture was transferred to the wells of a microtitre dish. Bacteria were transferred from the microtitre dish to a nylon membrane (Hybond N) using a replica plater (Sigma) and the inoculated membrane placed on the surface of a dried LB agar plate containing

15 ampicillin (100μg/ml). After overnight growth at 37°C, the membranes were treated with 1.5 M NaCl, 0.5 M NaOH for 10min. DNA was fixed to the membrane by microwaving at maximum power for 30s and bacterial debris removed by washing in 2XSSC. Pre-hybridisation and hybridisation were performed at 65°C in Rapid-hyb buffer (Amersham) for 1h and overnight, respectively. Blots were washed according

20 to standard procedures. To identify potentially attenuated mutants, hybridisation signals on a blot probed with labelled tags prepared from the input pool (inoculum) were compared to identical blots hybridised with labelled tags prepared from the corresponding output pools from at least two different animals.

25 3.   *Construction of DNA signature-tags and pLOF/TAG 1-48*

Double-stranded DNA signature tags were obtained by PCR amplification of the variable oligonucleotide pool RT1 (Hensel *et al*, 1995 Science 269 pp.400-403) using the following primers:

30

P3SAL (5'-CGCCATGTCGACCATTCTAACCAAGCTT-3')

P5SAL (5'-CGCCTAGTCGACTACAACCTCAAGCTT-3')

each of which contains recognition sites for the restriction endonuclease *Sal*I at its 5' terminus. To facilitate the cloning of the signature-tags in plasmid pLOF/Km, the

5 plasmid was modified to incorporate a unique SalI site. This was done by annealing the following complementary oligonucleotides:

SALTOP (5'-GTCGACCCT-3') and
SALBTM (5'-GTCGACAGG-3')

10

to give a double-stranded linker containing a *Sal*I site flanked by *Sfi*I-compatible ends. Plasmid pLOF/Km was digested with *Sfi*I, treated with calf intestinal alkaline phosphatase (CIAP), and ligated to the phosphorylated double-stranded linker to give plasmid pLOF/Sal. The PCR-amplified double-stranded DNA signature-tags

15 were digested with *Sal*I, gel purified, and cloned into the *Sal*I- digested, CIAP-treated, pLOF/Sal to generate a library of uniquely tagged pLOF/TAG plasmids.

Individual transformants in *E. coli* strain CC118 λ pir were screened by colony blot hybridisation with their corresponding [^{32}P]-dCTP-labelled tags to identify 100 tags

20 that amplified and labelled efficiently. These tags were then tested for an absence of cross-hybridisation and 48 plasmids (pLOF/TAG1-48) were selected for further use.

4.   *Construction of the signature-tagged mutant bank*

25

The 48 signature-tagged mini-Tn*10* transposons carried on plasmids pLOF/TAG 1-48 were separately introduced into *A. pleuropneumoniae* by conjugation. *A. pleuropneumoniae* strain 4074 Nal^R and *E. coli* S17 λ pir harbouring each of the pLOF/TAG 1-48 plasmids were grown overnight at 37°C to the stationary phase of

30 growth. Bacteria were harvested by centrifugation for 15 minutes at 3,000 rpm and washed twice with 10mM MgSO$_4$. Approximately equal numbers of *A.*

pleuropneumoniae recipient and *E. coli* donor cells were mixed, concentrated, and spread onto 0.22μM filters. Filters were placed onto BHI_L plates containing 1mM IPTG and incubated at 37°C with 5% CO_2 for 5 hours. Thereafter, the bacteria were removed into 5ml sterile PBS and aliquots plated onto BHI_L plates supplemented

5   with nalidixic acid (20μg/ml) and kanamycin (100μg/ml). Four separate matings were performed for each of the 48 signature-tagged mini-Tn*10* transposons. Transconjugant mutants were screened for susceptibility to ampicillin, thereby to eliminate strains carrying co-integrants of the suicide vector, inserted into the chromosome. In total 2064 transposon mutants from 48 individual matings were

10 isolated and arranged into 43 pools of 48 mutants. Mutants were stored in 1.5ml cryotubes with 15% glycerol at -80°C.

5.   Infection studies

15 Individual strains in each pool of 48 *A. pleuropneumoniae* signature-tagged mutants were grown separately until the mid-exponential phase of growth. At an OD_{600} of approximately 0.3, the individual mutant strains were combined and the pooled culture was diluted in HEPES to approximately 2×10^6 cfu/ml. Large White Cross piglets, of 6-10 weeks of age and determined to be *A. pleuropneumoniae* free by

20 bacteriological and serological analyses, were inoculated intra-tracheally with 3ml of the diluted culture. The number of cfu in the inoculum was verified by viable counts after plating serial dilutions of the inoculum on selective BHI_L. Each mutant pool was used to infect at least two animals.

25 At approximately 24h post-infection, surviving animals were euthanised and the lungs were removed. Surviving bacteria were recovered from infected lungs by either: (i) extensive lavage with Hanks buffered saline solution (HBSS); or (ii) homogenising the lungs in 500ml HBSS. Aliquots of the bacterial suspension were plated on selective media, and following growth overnight at 7°C with 5% CO_2,

30 approximately 10,000 colonies were pooled (the "output pool") and total chromosomal DNA was prepared.

6.   Competitive indices

To determine the *in vivo* competitive index (CI) of individual STM mutants, mutant
and wild-type bacteria were grown in Colombia broth with 5µg/ml NAD and 11 mM

5   CaCl$_2$ to an OD$_{600}$ of 0.3. The cultures were then combined to give equal numbers
of mutant and wild-type organisms, diluted to 2x10^6 cfu/ml; and inoculated intra-
tracheally into at least two animals. The ratio of mutant to wild-type bacteria in the
inoculum was verified by viable counts after plating serial dilutions of the inoculum
in parallel on BHI$_L$ + 20µg/ml nalidixic acid (mutant and wild-type), and BHI$_L$ +

10   20µg/ml nalidixic acid + 100µg/ml kanamycin (mutant only). Approximately 24h
post-infection, surviving animals were euthanised and the lungs were removed. The
lungs were homogenised in 500ml HBSS and aliquots were plated on selective
media (see above) to recover surviving bacteria. The *in vivo* CI was calculated as
the output ratio of mutant to wild-type organisms divided by the input ratio of mutant

15   to wild-type organisms. To determine *in vitro* CIs, mutant and wild-type organisms
were grown to mid-exponential phase, mixed in equal numbers, and the combined
culture incubated, with agitation, at 37°C for a further 3h. Samples were taken at
regular intervals, diluted, and plated on selective BHI$_L$ as before, and the *in vitro* CI
was calculated as described above.

20

7.   Virulence gene identification and DNA sequencing

To identify the *in vivo* attenuating lesions, DNA flanking the site of transposon
insertion was cloned or amplified by inverse PCR. For cloning, chromosomal DNA

25   was digested with a panel of restriction enzymes, separated by agarose gel
electrophoresis, transferred to a nylon membrane, and probed in a Southern blot
with a DIG-labelled probe specific for the Tn*10* kanamycin gene. Suitably sized
fragments hybridising to the probe were excised from a gel, purified and cloned into
appropriately digested pBR322. Transformants in *E. coli* strain XL1-Blue were

30   selected on LB agar containing 100µg/ml kanamycin.

To facilitate sequencing with the Tn*10* specific oligonucleotide primer:

BS401 (5'-GACAAGATGTGTATCCACC-3')

5   the cloned insert was further sub-cloned into plasmid pBCKS (Stratagene). Inverse PCR amplification of DNA flanking the site of transposon insertion was performed as described in Fuller, (2000) Microb. Pathog. 29, pp.25-38: chromosomal DNA was digested with *Ssp*I (which cuts once in the transposon), diluted to 150 ng/ml, and self-ligated for 15min using the Rapid DNA Ligation Kit (Roche). The ligated DNA

10   was then amplified using the primer pairs BS401 + TEF48, and BS401 + TEF62 (Fuller, 2000 Microb. Pathog. 29, pp.25-38). Reaction conditions for inverse PCR were as previously described (Fuller, 2000 Microb. Pathog. 29, pp.25-38). The resultant amplicons were purified following agarose gel electrophoresis using a QIAQUICK gel extraction kit (Qiagen). DNA sequencing reactions were performed

15   using the BigDye terminator cycle sequencing kit (Perkin-Elmer). Sequences from each of the attenuated mutants were used to query the non-redundant public DNA and protein databases using the BLAST set of similarity search programs (Altschul *et al* 1990 J. Mol Biol. 15, pp.403-410).

20   B.   <u>Results and Discussion</u>

1.   *Generation of signature-tagged mutants of A. pleuropneumoniae.*

The plasmid pLOF/Km, which is functional in *A. pleuropneumoniae*, was modified

25   to accept signature-tags by the insertion of a unique *Sal*I site in the mini-*Tn*10 element. PCR amplified random signature-tags were cloned into the *Sal*I site, and a bank of approximately 30,000 tagged plasmids in *E. coli* CC118 λ pir was obtained. A subset of these transformants was screened by colony blot with their corresponding radiolabelled tags, and signature-tagged plasmids with strong

30   hybridisation signals were selected. Plasmids with cross-hybridising tags were eliminated, and a pool of 48 unique signature-tagged plasmids was assembled.

These plasmids (pLOF/TAG 1-48) were transformed into *E. coli* S17 λ pir, and independently transferred to *A. pleuropneumoniae* strain 4074 Nal^R by conjugation.

Following the elimination of ampicillin-resistant plasmid cointegrants (0.5-1%), 2064
5 transposon mutants from 48 individual matings were isolated, and then arranged into 43 pools of 48 mutants. Southern blot analysis of ten randomly chosen exconjugants with a mini-Tn*10*-specific probe confirmed single, random insertion of the tagged transposon (data not shown). However, subsequent sequence analysis of attenuated clones revealed that mini-Tn*10* insertion in *A. pleuropneumoniae* was
10 not entirely random.

2.   *Signature-tagged screen of A. pleuropneumoniae mutants in pigs*

Signature-tagged *A. pleuropneumoniae* mutants were screened using a porcine
15 intra-tracheal infection model to identify genes involved in virulence. Bacteria were instilled directly into the trachea as previous studies have shown that aerosol particles produced by sneezing are small enough to penetrate into the lower respiratory tract, obviating the need for colonisation of the upper respiratory tract (Kaltreider HB. in Immunologic and infectious diseases in the lung. (1976) pp. 73-97,
20 Kirkpatrick & Reynolds (Eds) Marcel Dekker, New York).

A range of experimental factors including the infecting dose, and the method of recovery were investigated before we could reproducibly recover the majority of individual mutants following *in vivo* selection in the porcine host. Using challenge
25 doses of 10^3, 10^4, or 10^5 cfu, for example, there was a poor correlation between the infecting dose and the appearance of clinical or morphological signs of disease. Moreover, when different pigs were infected with the same pool, the hybridisation patterns of the post-passage pool suggested that different clones were missing after passage of a given pool through different animals (data not shown). This
30 inconsistency was overcome by increasing the challenge dose to 10^6 - 10^7 cfu/pig.

In an earlier STM study of *A. pleuropneumoniae* infection in pigs [Fuller, 2000 Microb. Pathog. 29, pp.39-51], bacteria were recovered by lung lavage following experimental infection. However, this method was found to be unreliable, yielding essentially random recovery of individual signature tags. In contrast, we observed
5   consistent recovery of viable bacteria following homogenisation of the entire lung in HBSS. Optimisation of these two crucial parameters resulted in the recovery of the majority of individual clones (>90%, 20-48h post infection), with excellent reproducibility between animals (see Figure 1; compare A and B).

10 In total, 2064 transposon mutants (43 pools of 48 mutants) were screened for attenuation *in vivo*, each pool being inoculated into at least two different animals. Surviving bacteria were recovered by homogenisation of lungs in sterile HBSS. This initial screen identified 550 mutants present in the input pool but missing or significantly reduced in the output pools. These potentially attenuated mutants were
15 reassembled into new pools and each new pool was used to infect a further two animals. Following this second screen, 105/410 mutants were found to be consistently absent from the output pools, and these were retained for further analysis.

20 3.   *Identification and analysis of A. pleuropneumoniae virulence genes.*

The nucleotide sequence of the DNA flanking the site of transposon insertion was obtained for each of the 105 attenuated mutants as described, and used to search the GenBank databases for homologous genes. The results of this analysis are
25 shown in Table 1, with the genes assigned to one of five functional categories: cell surface, transport, metabolism, stress responses, and regulation. Insertions in genes encoding proteins with no significant homology to proteins in the databases, or homologous to proteins or predicted proteins of unknown function, are categorised as "unknown". The 105 attenuated mutants contained transposon
30 insertions in 53 individual genes and a number of hotspots for Tn*10* insertion were identified. In particular, seven identical insertions were independently isolated in the

putative *rfbP* gene (*Tn* insertion at codon 444),3 of 4 insertions in the *A. pleuropneumoniae fur* gene were located at codon 128. Interestingly, the DNA sequences flanking the 3 hotspots which were identified in this study are neither similar to each other, nor to the consensus sequence for Tn*10* insertion into *A.*

5   *pleuropneumoniae* that has been previously been identified (Fuller et al (2000) Microb. Pathog. 29, pp.25-38).

(i)   <u>Cell surface</u>

10   Mutations in capsule and lipopolysaccharide (LPS) genes are known to attenuate *A. pleuropneumoniae* (Rioux *et al.*, Can. J Microbiol. 45: pgs1017-1026, 1999) (Rioux *et al.*, Microb Pathog 28: pgs 279-289, 2000; Ward *et al.*, Infect Immun 66: pgs 3326-3336, 1998). Mutant 9C2 contains a transposon insertion in the *cpxC* gene that encodes a protein involved in capsule export in *A. pleuropneumoniae*

15   (Ward and Inzana, 1997). This mutant was used as a control (known attenuated) strain the *in vivo* CI experiments. Five genes involved in the synthesis of O-antigen of *A. pleuropneumoniae* serotype 1 were identified in this STM screen. Mutants with inserts in genes 9 (mutant 10B11), 12 (mutant 25B7), 15 (mutant 15A9), 17 (mutant 12D5), and 18 (mutant 21B8) of the O-antigen operon (Labrie *et al.*, 2002, J.

20   Endotox Res. 8: 27-38) were all attenuated. Another mutant, 9B7, has the transposon insertion 20 bases upstream of the *A. pleuropneumoniae galU* gene that encodes a protein involved in synthesis of the LPS core. Mutation of *galU* was previously shown to attenuate *A. pleuropneumoniae* (Rioux *et al.*, 1999). Finally, mutant 26A9 contains an insertion in a gene homologous to *lcbB*, a putative

25   bifunctional polymerase implicated in capsule or LPS biosynthesis in *N. meningiditis* serogroup L.

Strain 4D4 contains a transposon insertion in a gene encoding a putative lipoprotein. Strain 4D4 shows greatest homology to an unknown protein from *P. multocida*, but

30   it is similar to lipoproteins and putative lipoproteins of *H. influenzae* and *H. somnus* [Theisen (1993) Infect Immun 61: 1793-1798]. *H. somnus* LppB binds Congo red

- 33 -

dye (a structural analogue of heme) and has been suggested to be a virulence factor, but this has not been formally proven [Theisen (1993) Infect Immun 61: 1793-1798]. Finally, strain 17A4 contains an insertion in an ORF with homology to the P2 porin protein of *H. influenzae* [Duim et al (1993) Microb. Pathog. 14, pp. 451-462].

5   The P2 protein constitutes approximately one half of the total outer membrane protein of *H. influenzae* and represents an important target of the immune response to non-typeable *H. influenzae* infection. [Yi et al (1997) Infect. Immun. 65, 150-155].

(ii)   Metabolism

10

Strain 10B12 contains a transposon insertion in an ADP-ribose pyrophosphatase (*adpP*) gene homologue. Regulation of cellular levels of ADP-ribose (ADP-ribose is derived from NAD) is important in preventing non-enzymatic ADP-ribosylation of proteins. The *E. coli,* ADP-ribose pyrophosphatase catalyses the hydrolysis of ADP-

15   ribose to ribose-5-P and AMP, compounds that can be recycled as part of nucleotide metabolism [Gabelli et al (2001) Nat Struct. Biol. 8, pp. 467-472].

Strain 10A11 carries an insertion in an argininosuccinate synthase, *argG,* gene homologue. Argininosuccinate synthase catalyses the conversion of citrulline and

20   aspartate into argininosuccinate, linking the urea cycle and the citric acid cycle via the so-called aspartate-argininosuccinate shunt. The aspartate-argininosuccinate shunt provides a metabolic link between the pathways by which amino groups and the carbon skeletons of amino acids are processed. (Glansdorff (1996) Biosynthesis of arginine and polyamines. In *Escherichia coli and Salmonella* (Vol. 1) (Neidhardt,

25   F.C. et al., eds), pp. 408-433, ASM Press).

In strain 33C7, the attenuating lesion was found to occur in a gene homologous to *H. influenzae atpA*, encoding the F_1 alpha subunit of F_1F_0 ATP synthase. ATP synthase is responsible for utilising the proton-electrochemical gradient across the

30   cytoplasmic membrane for ATP synthesis in cells growing aerobically or anaerobically. All eight subunits of the bacterial enzyme are encoded by the *atp*

operon. In *P. multocida* the *atpA* gene is flanked by *atpH* and *atpG*, and while the gene order in *A. pleuropneumoniae* is unknown, both *atpH* and *atpG* have been implicated previously in the pathogenesis of *A. pleuropneumoniae* infection [Fuller (2000) Microb. Pathog. 29, pp.39-51].

5

In strain 0A7, the transposon has inactivated the *pntB* gene, encoding the beta subunit of NAD(P) transhydrogenase. Transhydrogenase is an integral membrane protein composed of two subunits, α and β, organised as an $\alpha_2\beta_2$ tetramer. Transhydrogenase couples the redox reaction between NAD(H) and NADP(H) to the

10 translocation of protons across the inner membrane (Penfound & Foster (1996) Biosynthesis and recycling of NAD. In *Escherichia coli and Salmonella* (Vol. 1) (Neidhardt, F.C. et al., eds), pp. 408-433, ASM Press). It plays a key role in energy metabolism, biosynthesis and detoxification.

15 In strain 19D5, the transposon has inserted in the *A. pleuropneumoniae mrp* gene. The partial *A. pleuropneumoniae mrp* gene sequences available are highly homologous to *mrp* genes from *H. influenzae* and *E. coli*. In *H. influenzae*, the *mrp* gene has been implicated in the biosynthesis of the Gala(1-4)βGal component of LPS, while in *E. coli* mutations in *mrp* affect the synthesis of thiamine [High et al

20 (1996) FEMS Microbiol. Lett. 145, pp.325-331; Petersen (1996) J. Bacteriol. 178, pp. 5676-5682]. In *A. pleuropneumoniae*, the *mrp* gene is located approximately 1kb upstream from the *apxIVA* gene, encoding the *in vivo*-induced RTX toxin, ApxIV [Schaller (1999) Microbiology 145: 2105-2116].

25 Strain 29B11 contains a transposon insertion in a *napB* gene homologue, encoding the smaller, dihaem cytochrome c, subunit of heterodimeric periplasmic nitrate reductase (NAP). In *H. influenzae* (and Pseudomonas *sp*) NAP is the sole nitrate reductase and may function to support anaerobic growth in the presence of nitrate [Brige (2001) Biochem. J. 356, pp. 851-858; Fleischmann (1995) Science 269, pp.

30 496-512; Bedzyk (1999) J Bacteriol 181: 2802-2806]. In *E. coli* the situation is more complex with two membrane bound respiratory nitrate reductases in addition to NAP

[Moreno-Vivian (1999) J Bacteriol 181: 6573-6584]. There is evidence to suggest that NAP is a dissimilatory enzyme used for redox balancing. In addition, NAP has been suggested to play a role in adaptation to anaerobic metabolism after transition from aerobic conditions, the utilisation of alternate reductants, or even as a self-
5 defense mechanism forming high nitrite levels to inhibit the growth of competing bacteria [Moreno-Vivian (1999) J Bacteriol 181: 6573-6584]. Consistent with a role in anaerobic adaptation, transcription of the *Salmonella typhimurium napB* gene is induced, in an Fnr-dependent manner, in cells exposed to anaerobic conditions [Wei and Miller (1999) J. Bacteriol. 181, pp. 6092-6097].

10

Strain 17B8, contains an insertion in a gene homologous to *H. influenzae ccmH*, required for the assembly of C-type cytochromes, including NAP, in enteric bacteria [Thony-Meyer (1995) J. Bacteriol. 177, pp. 4321-4326; Kranz (1998) Mol Microbiol 29, pp. 383-396]. CcmH is encoded by the final gene of the *ccm* operon and with
15 CcmF and CcmG is believed to catalyse the reduction of disulphide bonds in the cytochrome *c* apoprotein and to facilitate haem attachment [Kranz (1998) Mol Microbiol 29, pp. 383-396]. While in E. coli the *nap* and *ccm* operons are contiguous and may be cotranscribed [Grove (1996) Mol Nicrobiol 19, pp. 467-481] they are located at separate loci in both *H. influenzae* and *P. multocida* [Fleischmann (1995)
20 Science 269, pp. 496-512; May (2001) Proc Natl Acad Sci USA 98, pp. 3460-3465].

Strain 4B9 contains a transposon insertion in a *moaA* gene homologue. In *E. coli* the *moaA-E* genes are required for the synthesis of molybdopterin from GTP [Rivers (1993) Mol Microbiol 8: 1071-1081]. Molybdopterin (molybdopterin guanine
25 dinucleotide) is the principal cofactor found in prokaryotic molybdoproteins including periplasmic nitrate reductase. Expression of the *moa* genes is induced by anaerobiosis [Anderson (2000) J. Bacteriol. 182, pp. 7035-7043] and *Salmonella typhi moaA* mutants are impaired in their ability to replicate within epithelial cells [Contreras (1997) Microbiology 143, pp. 2665-2672].

30

Mutant 29B12 contains an insertion in the *A. pleuropneumoniae recR* gene

homologue. The RecR protein is involved in the RecF pathway of recombinatorial DNA repair: *recR* mutants are deficient at filling in daughter strand gaps in newly synthesised DNA [Kuzminov (1999) Microbiol Mol Biol Rev 63, pp. 751-813]. Mutations in *recR* delay induction of the SOS response in *E. coli* and such mutants

5   are sensitive to UV-irradiation and mitomycin C [Keller (2001) Mutat Res 486, pp. 21-29].

In strain 26A10, the transposon has inserted in a gene with extensive homology to *thrC* of *H. influenzae*. The *thrC* gene encodes threonine synthase, the final enzyme

10 in the biosynthetic pathway leading to the production of threonine (Patte (1996) Biosynthesis of threonine and lysine. In *Escherichia coli and Salmonella* (Vol. 1) (Neidhardt, F.C. et al., eds), pp. 408-433, ASM Press). Mutations in a *Streptococcus pneumoniae thrC* homologue attenuate the virulence of this organism in a murine model of pneumoniae [Lau (2001) Mol Microbiol 40, pp. 555-571].

15

In strain 26D12, the transposon has inactivated a GMP synthase, *guaA*, gene homologue. GMP synthase catalyses the synthesis of GMP from XMP as part of the purine biosynthetic pathway. Mutations in the *guaBA* operon attenuate the virulence of a variety of enteric pathogens including *E. coli, S. flexneri, and S. typhi*, and a

20 Δ*guaBA S. typhi* derivative shows promise as a live attenuated vector [Wang (2001) Infect Immun 69, pp. 4734-4741; Noriega (1996) Infect Immun 64, 3055-3061; Russo (1996) Mol Microbiol 22, pp. 217-229]. Moreover, previous STM screens in *Streptococcus agalactiae* and *P. multocida* have implicated *guaA* and *guaB*, respectively, in growth *in vivo* and in pathogenesis [Jones (2000) Mol Microbiol 37,

25 pp. 1444-1455; Fuller (2000) Microb Pathog 29, pp. 39-51].

Mutant 27A12 contains a transposon insertion in a gene previously identified in *A. pleuropneumoniae* as *tonB* (Tonpitak *et al.*, Infect Immun 68: 1164-1170 2000) (now referred to as *tonB1*). This gene is found immediately upstream of, and is be

30 co-transcribed with, *exbB, exbD,* and the transferrin binding protein (*tbp*) genes. In contrast, strain 0F6 contains a transposon insertion in an ORF encoding a protein

with homology to TonB proteins from *P. multocida* and *Haemophilus sp.* (now designated *tonB2*). Moreover, sequence analysis of the region upstream from the transposon insertion in OF6 revealed the presence of distinct *exbB* and *exbD* gene homologues, suggesting that *A. pleuropneumoniae*, in common with other bacterial

5   pathogens including *V. cholerae* and *P. aeruginosa*, possesses two independent TonB systems (Occhino *et al.*, Mol Microbiol 29: 1493-1507 1998; Zhao and Poole, FEMS Microbiol Lett 184: 127-132 2000).

Strain 35D11 contains an insertion in a gene homologous to *H. influenzae dsbA*.
10   DsbA is a small periplasmic protein and a member of the thioredoxin superfamily [Debarbieux (1999) Cell 99, pp.117-119]. Mutations in *dsbA* are pleiotropic, affecting the production of a variety of translocated proteins including bacterial virulence factors, components of type III secretion machinery, and c-type cytochromes including NAP [Yu and Kroll(1999) Microbes Infect 1, pp. 1221-1228]. Mutations in
15   *dsbA* and *dsbB* have been implicated in the virulence of *Shigella flexneri* [Yu (1998) Infect Immun 66, 3909-3917] and *P. multocida* [Fuller (2000) Microb Pathog 29, pp. 25-38], respectively.

In strain 26C3, the transposon has inactivated a glutamyl-tRNA reductase, *hemA*,
20   gene homologue while in strain 26D5 the transposon has inserted in a putative uroporphrinogen decarboxylase *uroD/hemE* gene homologue. Glutamyl-tRNA reductase catalyses the reduction of glutamate to glutamate 1-semialdehyde, the first committed step unique to the formation of δ-aminolevulinate (ALA) from glutamate. Downstream from ALA formation, uroporphrinogen decarboxylase
25   catalyses the decarboxylation of uroporphrinogen III to yield coproporphrinogen, the first step in the conversion of uroporphrinogen III to protoheme via protoporphyrin IX. Hemes, including protoheme, are key components of the electron transfer apparatus and in addition play important roles as enzyme prosthetic groups in mineral nutrition and oxidative catalysis (Beale, Biosynthesis of hemes. In
30   *Escherichia coli and Salmonella* (Vol. 1) (Neidhardt, F.C. et al., eds), pp. 408-433, ASM Press).

In strain 15A11, the transposon has inserted into a gene homologous to *P. multocida visC*. The protein encoded by *visC* encodes a putative monooxygenase belonging to the. UbiH/CoQ6 family. UbiH is required for the biosynthesis of ubiquinone [Nakahigashi (1992) J. Bacteriol. 174, pp. 7352-7359], a prenylated

5 benzoquinone that functions in the respiratory electron chain in the plasma membrane of prokaryotes. Ubiquinone and protoheme also function in the formation of disulfide bonds in periplasmic proteins by facilitating the oxidation of DsbA by DsbB [Kobayashi (1997) Proc Natl Acad Sci USA 94, pp. 11857-11862].

10 Strain 29A10 contains a transposon insertion in a gene homologous *to prfC* of *H. influenzae*. The *prfC* gene encodes peptide chain release factor 3 (RF3), a GTPase that in the presence of GTP catalyses the removal of RF1 and RF2 from the ribosome after translation termination [Freistroffer (1997) EMBO J 16 4126-4133]. The efficient recycling of RF1 and RF2 by RF3-GTP, although dispensable for

15 viability in prokaryotes, is required for maximum growth rate [Kisselev (2000) Trends Biochem Sci 25, pp. 561-566].

In strains 26A6 and 33B7 the transposon has inserted in genes with homology to the *trmH* family of tRNA/rRNA methyltransferases. The interrupted ORF in strain

20 26A3 shows greatest homology to *H. influenzae yibK*, while that in strain 33B7 shows most similarity to *yjfH* of *H. influenzae*.

(iii)   Regulation

25 Virulence genes involved in environmental sensing and coordinate regulation were identified in this screen. The *A. pleuropneumoniae* homologue of the ferric uptake regulator, *fur*, was found to be inactivated in strain 6C12. Fur or Fur-like proteins have been shown to regulate virulence factor expression in a variety of pathogens including *E. coli* (Shiga-like toxin), *Corynebacterium diptheriae* (diptheria toxin), and

30 *Pseudomonas aeruginosa* (exotoxin A) [Prince (1991) Mol. Microbiol 5 pp.2823-2831; Schmitt (1991) Infect Immun 59, pp. 1899-1904; Calderwood (1987) J.

Bcateriol 169, pp. 4759-4764]. Strains 21D3 and 19B10 contain transposon insertions in the A. pleuropneumoniae rpoE and upstream mclA (rseA) gene homologues, respectively. The rpoE encoded sigma factor, σ^E/Sigma-24, is a member of the extracytoplasmic function subfamily of sigma factors that function as

5 effector molecules in response to extracytoplasmic stimuli including heat shock and oxidative stress [Raina (1995) EMBO J 14, pp. 1043-1055; Rouviere (1995) EMBO J. 14 1032-1042; Ades (1999) Genes Dev 13, pp. 2449-2461; Dartigalongue (2001) J Biol Chem 276, pp. 20866 - 20875]. In S. typhimurium, σ^E is an important determinant of virulence and immunogenicity [Humphreys (1999) Infect Immun 67,

10 pp. 1560-1568]. MclA (RseA) is an inner membrane protein that acts as a σ^E-specific anti-σ factor. MclA (RseA) is rapidly degraded in response to extracytoplasmic stress resulting in increased σ^E concentration and initiation of the stress response [Ades (1999) Genes Dev 13, pp. 2449-2461]. Finally, insertions in the A. pleuropneumoniae quorum sensing luxS homologue (strain 26D3) were

15 observed to attenuate A. pleuropneumoniae virulence. Recently, the E. coli luxS homologue has been implicated in the control of intestinal colonisation factors encoded by the locus of enterocye effacement pathogenicity island in both EHEC and EPEC [Sperandio (2001) J Bacteriol 183, pp. 5187-5197; Sperandio (1999) Proc Natl Acad Sci USA 96, pp. 15196 - 15201].

20

(iv)   <u>Transport</u>

The A. pleuropneumoniae gene aopA is inactivated in strain 23C9. The aopA gene encodes a 48 kDa immunogenic outer membrane protein that is common to all

25 serotypes of A. pleuropneumoniae tested [Cruz (1996) Infect Immun 64 pp. 83-90]. Interestingly, AopA is highly homologous (75% identity, 84% similarity) to Nqr1 of H. influenzae (NqrA of Vibrio alginolyticus), encoding the alpha chain of the sodium-translocating NADH-ubiquinone oxidoreductase (NQR) complex [Hayashi (1996) FEBS Lett 381 pp. 174-176; Beattie (1994) FEBS Lett 356 pp. 333-338]. The NQR

30 enzyme is a respiration-linked Na^+ pump establishing an electrochemical gradient of sodium ions across the membrane. The resultant sodium motive force can be

used for solute transport, ATP synthesis, and flagellar rotation. This alternative energy coupling of sodium ions rather than protons enables the bacteria to maintain a cytoplasmic pH near neutrality in an alkaline environment. The NQR enzyme has been most extensively studied in *V. alginolyticus* where it is thought to be involved

5   in pH and ion homeostasis [Beattie (1994) FEBS Lett 356 pp. 333-338; Unemoto (1993) J. Bioenerg Biomembr 25 pp. 385-391]. In *Vibrio cholerae*, the NQR complex has been implicated in the regulation of virulence factor expression via its effects on ToxT expression [Hase (1999) Proc Natl Acad Sci USA 96 pp. 3183-3187].

10 Components of several different putative ABC transport systems were identified as necessary for virulence of *A. pleuropneumoniae*. ABC transport systems are involved in the transport (uptake or efflux) of a diverse array of macromolecules across the cytoplasmic membrane of bacteria and eukaryotes. These systems usually consist of three basic parts: one or two ATPases, one or two integral

15 membrane proteins and a substrate-specific binding protein. Strains 13B12, 0C5, 19D1, and 24A4 contain transposon insertions in the ATPase component of ABC transporters. The gene inactivated in strain 0C5 encodes a protein with significant homology to MgIA of prokaryotic galactose transporters (Richarme *et al.*, J Biol Chem 268: 9473-9437, 1993). The *in vivo* CI of this mutant indicates limited

20 availability of certain sugars within the respiratory tract environment. The interrupted ORFs in strains 19D1 and 24A4 are homologous to hypothetical ATPases from transport systems of unknown specificity in *P. multocida* (PM1728 and PM0996, respectively). In strain 20D10, the transposon has inactivated a gene with extensive homology to *znuA* of *H. ducreyi* and *pzp1* of *H. influenzae* (Lewis *et al.*, Infect

25 Immun 67: 5060-5068, 1999; Lu *et al.*, J Biol Chem 272: 29033-29038 1997 and FEMS Microbiol Lett 165: 129-137 1998). ZnuA (PZP1) is a periplasmic zinc-binding protein that plays a key role in zinc uptake. Inactivation of the *znuA* gene in *H. ducreyi* caused a significant decrease in virulence in the rabbit model for experimental chancroid (Lewis *et al.*,Infect Immun 67: 5060-5068, 1999). In strain

30 0D6 the transposon has inserted in a gene encoding a protein with homology to putative integral membrane proteins from *P. multocida* (PM0997) and *N.*

meningitidis (NMB0549). In *P. multocida* PM0997 is located immediately upstream from the ATPase inactivated in strain 24A4 suggesting that they are part of the same transport system (May *et al.*, Proc Natl Acad Sci U S A 98: 3460-3465 2001). This gene was also identified by Fuller *et al.* (Fuller *et al.*, Microb Pathog 29: 39-51

5 2000) as *apvD*, the predicted product of which shows homology with the macrolide-specific ABC-type efflux protein MacA (Kobayashi *et al.*,J Bacteriol 183: 5639-5644 2001). In mutant 9D10, the disrupted gene sequence encodes a protein with homology to CorC which mediates both influx and efflux of magnesium as well as cobalt efflux (Gibson et al., Mol Microbiol., 5: pgs 2753-2762,[1991]).

10

(v)   **Stress**

In strain 13A3, the transposon has inserted in a *dnaJ* gene homologue. DnaJ is an essential component of bacterial Hsp70 chaperone systems where it functions as

15   a co-chaperone, providing substrate specificity to its Hsp70 partner, DnaK [Rudiger (2001) EMBO J 20 pp. 1042-1050]. In addition to their fundamental roles in protein folding and translocation, DnaK/DnaJ constitute the primary stress-sensing and transduction system of the *E. coli* heat shock response [Tomoyasu (1998) Mol Microbiol 30 pp.567-581] where they modulate the induction of the heat shock

20   response by altering the stability of the heat shock sigma factor σ^{32}. Significantly, mutations in DnaK have previously been shown to attenuate *A. pleuropneumoniae* virulence and to decrease the survival of *Brucella suis* in human macrophage-like cell lines [Fuller (2000) Microb Pathog 29 pp. 39-51; Kohler (1996) Mol Microbiol 20 pp. 701-712].

25

Strain 26B6 contains an insertion in a *htpG* gene homologue. HtpG is a heat shock-induced molecular chaperone that, in *E. coli*, contributes to the correct folding of cytoplasmic proteins in mildly stressed cells [Thomas (2000) Mol Microbiol 36 1360-1370]. HtpG does not appear to associate stably with partner proteins and little is

30   known about its mechanism of action. It has been suggested that HtpG serves as a holder chaperone, transiently maintaining a subset of de novo synthesised

proteins in a conformation that is accessible to DnaK/DnaJ [Freeman (1996) EMBO J 15 pp.2969-2979].

Strain 13D8 contains a transposon insertion in a *lon* gene homologue. Lon (also
5 called protease La) is a heat shock-inducible, ATP-dependent serine protease that plays a major role in the elimination of abnormally folded proteins [Gottesman (1996) Annu Rev Genet 30 465-506; Suzuki (1997) Trend Biochem Sci 22 pp.118-123]. In addition to its demonstrated role as a stress response protease, Lon also functions as a pleiotropic regulator of gene expression by degrading unstable
10 regulatory proteins including RcsA, a positive regulator of capsule synthesis [Keenleyside (1992) J Bacteriol 174 pp. 8-16] and SulA, an inhibitor of cell division and component of the *E. coli* SOS response [Mizusawa (1983) Proc Natl Acad Sci USA 80 pp. 358-362]. Recent studies in *Brucella abortus* have demonstrated that Lon is required for survival in murine macrophages and wild-type virulence in the
15 mouse model of infection [Robertson (2000) Mol Microbiol 35: 577-588].

Strain 13C1 carries a transposon insertion in a *prc* gene homologue. Prc is a periplasmic protease whose natural substrates appear to be membrane proteins including TonB [Gottesman (1996) Annu Rev Genet 30 pp.465-506]. It has been
20 speculated that Prc functions as a chaperone involved in the folding and turnover of proteins in the periplasmic space [Bass (1996) J Bacteriol 178 pp.1154-1161].

CLAIMS

1.   An attenuated *Actinobacillus pleuropneumoniae* bacterium.

2.   An attenuated *Actinobacillus pleuropneumoniae* bacterium having a mutation in a gene required for bacterial virulence.

3.   An attenuated *Actinobacillus pleuropneumoniae* bacterium having a mutation in a gene which comprises a nucleotide sequence selected from the group consisting of SEQ ID NO.:1-56.

4.   An attenuated *Actinobacillus pleuropneumoniae* bacterium having a plurality of mutations, occurring within a single gene or within different genes.

5.   A composition containing an attenuated *A. pleuropneumoniae* bacterium according to any previous claim.

6.   A composition according to Claim 5, comprising a plurality of different attenuated *A. pleuropneumoniae* bacteria, having different mutations in the same virulence gene and/or bacteria having similar, or different, mutations in two or more different genes.

7.   A composition according to any one of Claims 5 or 6, further comprising one or more pharmacologically acceptable adjuvants, diluents, carriers, vehicles and/or excipients.

8.   A composition according to any of Claims 5 -7, which is packaged in a form suitable for administration by intravenous, intradermal, intramuscular, intramammary, intraperitoneal and/or subcutaneous injection, and/or by oral,

sublingual, nasal, anal or vaginal delivery.

9.   A composition according to any of Claims 5 -8, which is selected from the group consisting of an immunogenic composition, a therapeutic (e.g. prophylactic) composition and a vaccine.

10.   A composition according to Claim 9, wherein the composition is a vaccine whose administration to an animal confers a degree of cross-protection.

11.   Use of an attenuated A. pleuropneumoniae bacterium according to any of Claims 1-4 in the manufacture of a medicament for preventing or alleviating an infection of an animal with A. pleuropneumoniae.

12.   Use of an attenuated A. pleuropneumoniae bacterium according to any of Claims 1-4 in the manufacture of a medicament for preventing or alleviating symptoms associated with A. pleuropneumoniae infection, such as the prophylactic protection of swine against porcine pleuropneumonia.

13.   An isolated polynucleotide encoding a gene product which is naturally involved in (e.g. required for) the virulence of A. pleuropneumoniae.

14.   An isolated polynucleotide encoding a gene product which is not naturally found in A. pleuropneumoniae, but whose expression therein is capable of modulating (e.g. of decreasing) the virulence of that bacterium.

15.   An isolated polynucleotide which is not naturally found in A. pleuropneumoniae but which is capable of modulating the virulence of that bacterium by its direct interaction with A. pleuropneumoniae virulence genes or gene products.

16.   An isolated polynucleotide comprising: (a) a nucleotide sequence selected

from the group consisting of SEQ ID NO.: 1-56; (b) a nucleotide sequence encoding the polypeptide which is encoded by the nucleotide sequence recited in (a); (c) a nucleotide sequence which hybridizes to the nucleotide sequence of (a) and/or (b), or to its complement, under conditions of

5   moderate to high stringency; or (d) a fragment of any one of the nucleotide sequences of (a)-(c), which fragment retains an immunological property and/or a biological activity of the recited nucleotide sequence of (a)-(c).

17.   A vector comprising a polynucleotide according to any of Claims 13-16.

10

18.   A host cell containing a polynucleotide or vector according to Claims 13-17.

19.   An attenuated bacterium containing a mutation in a gene comprising a nucleotide sequence which is capable of hybridising to any one of the

15   nucleotide sequences defined by SEQ ID NO:1-56, under conditions of moderate to high stringency.

20.   An attenuated bacterium according to Claim 19, having a plurality of mutations, occurring within a single gene or within different genes.

20

21.   A composition containing a bacterium according to Claims 19 or 20.

22.   A composition according to Claim 21, comprising a plurality of different attenuated bacteria, having different mutations in the same virulence gene

25   and/or bacteria having similar, or different, mutations in two or more different genes.

23.   A composition according to any one of Claims 21 or 22, further comprising one or more pharmacologically acceptable adjuvants, diluents, carriers,

30   vehicles and/or excipients.

24. A composition according to any of Claims 21-23, which is packaged in a form suitable for administration by intravenous, intradermal, intramuscular, intramammary, intraperitoneal and/or subcutaneous injection, and/or by oral, sublingual, nasal, anal or vaginal delivery.

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25. A composition according to any of Claims 21-24, which is selected from the group consisting of an immunogenic composition, a therapeutic (e.g. prophylactic) composition and a vaccine.

10   26. A composition according to Claim 25, wherein the composition is a vaccine whose administration to an animal confers a degree of cross-protection.

27. Use of a bacterium according to Claims 19 or 20 in the manufacture of a medicament for the therapeutic treatment or prophylactic protection of an

15   animal against infection by the corresponding wild-type bacterium (or a different strain or serotype thereof).

28. An isolated A. pleuropneumoniae virulence polypeptide.

20   29. A method of producing a virulence polypeptide according to Claim 28, comprising the steps of: (i) culturing a host cell according to Claim 18 under conditions that permit the expression of the polypeptide; and (ii) recovering and optionally isolating the expressed polypeptide from the host cell, or from its surrounding medium.

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30. A virulence polypeptide encoded by a polynucleotide according to any of Claims 13-16.

31. A composition comprising a polypeptide according to Claim 28 or 30.

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32. A composition according to Claim 31, further comprising one or more

pharmacologically acceptable adjuvants, diluents, carriers, vehicles and/or excipients.

33.   An antibody which specifically recognizes a polynucleotide or polypeptide according to any of Claims 13-16 and Claim 28 or 30.

34.   A method for identifying an anti-bacterial agent which is capable of modulating the function of an *A. pleuropneumoniae* virulence gene of the present invention, or of a homologous gene in a related species.

35.   A method according to Claim 34, comprising screening potential agents for their ability to interfere with the expression and/or biological activity in a host bacterium of the gene products encoded by the nucleotide sequences set forth in any one of SEQ ID NO:.1-56.

36.   An agent identified by the method of Claims 34 or 35.

37.   A method of modulating the transcription of such virulence genes through the use of oligonucleotide-directed triplet helix formation.

38.   A composition comprising an anti-bacterial agent according to Claim 36.

39.   A method of treating an animal suffering from a *Pasteurellaceae* (e.g. an *A. pleuropneumoniae*) infection by administration of an anti-bacterial agent or composition according to any of Claims 36 or 38.

40.   Use of an agent or composition according to Claims 36 or 38 in the manufacture of a medicament for the treatment of a *Pasteurellaceae* infection and/or porcine pleuropneumonia.

BACTERIAL VIRULENCE GENES

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ABSTRACT

10 An attenuated *Actinobacillus pleuropneumoniae* bacterium has a mutation in a gene required for bacterial virulence. Vaccines based upon the bacterium are provided, as are isolated virulence genes and polypeptides and uses thereof.

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AAGAGCAACATAATATCTGGATTGTCGGCACGGCAGGTGAAGCGACTTCCGGTATTTATCAGGCT
AAATTAACCGGTGCTATCGCt

SEQ ID No. 17

ATTGCGCAAAATGCTAACAGAATTATCAAAATCAAAGATGGCGAAATTAT
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CCAATCCGTTTCTTAAATAAAACTGCCAATGCAACGATAAATGGTGTTAG
CCAAGATTTCTTTATGTTAAAAAACTACAAATTACTAAGTGGCAGATTTT
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AAATCACGAGATGTAATCTTCGGCACAAAAATTGCAGAAAGCAATATAGT
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GTTACGAGAAAATTTTGTACCAGCAGCAGCCGATAAGGCTATTGTGGATT
TATTGGCAGCAAAGCAATCCATATCATGGCAGATAGGATTTTTTTACTTT
CAGTAGCAGCAAGTTCTTGCAATCCTTAAATCGCACTACACAGCCCTAAC
TTTAATGATTTCATCTATCGCTTTTATTTCGCTGGTAGTTGGAGGTATAG
GAGTTATGAAT

SEQ ID No. 18

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GGTTTGATATAAGGTAAACTTATAGAAATTTTTCACGTCTAACCATAGCA
AAAAAGCGATAAAACTGGTAATTTGACTATTCGTATCTGAAAACTATTCT
TATTTTTAATTTAAACGGGATGACAATGTTATTTGCGATTTTAGCAATGC
TGGCTTATGCTGCCGCATTATTATGGGTTACCCCCACACTCGTCAATTTA
GAAAACCAAACCGGTGATAAAAAACCGAATCTTAAAGCGGTTTTTGGATT
CGGCTTACTTGCCGTATTCTTTCACGCAATCAATTTATCGCAAGAATTGG
TGCTGAGCGTGCTGAGCGAGGGCGGTCAGAATTTCTCACTTTCTAACGTA
AACTCAATGATGAGCTTATTGCTGACGGCTGTCGCAACACTTGCCTTACC
GCGTTGGAAAACCATTTGGTTTCCGTTAATGGTGGTTTATACCTTCGGCA
TTTGTAGCGTAGCGGCATCCGCATTTGCCAGCGGTAACTTTATTAAAAAT
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SEQ ID No. 19

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GCAATACCTGCACTCGGGCCGTCTTTCGGCGTTGCGCCGTCCGGCACGCG
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ACATCGCCTAATGAACCGGTAAATGAGAATTTACCTTTACCGCCGACCGA
AGTGGTTTCGATGGTGAGTAAATCGCCGCCGACTTCCGTCCAAGCTAAGC
CGGTTACTTCACCGACACGGTTTTGGCTGTCCATTTTACCGTAATCAAAA
CGTTTTACACCCAAGTAATCGGCGATATTGCCTTCGGTTACTGTAATTGA
TTTGACTTTTTTGTCCAACACCAGAGTTTTCACCGCTTTACGACAGATCT
TAGCAATTTCACGTTCAAGACTACGCACACCAGCTTCACGAGTGTAGTAA
CGAATAATGCTTAAAATAGCACTATCTTCGATAGTTAATTCACCCGCTTT
TAAGCCGTTATTTTCTTGCTGTTTGGCAATTAAGTGGTCACGGGCAATGT
GCATTTTCTCATCTTCGGTATAACCGGAAAGACGAATCACTTCCATACGG
TCGAGTAATGCCGGCGGAAT
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SEQ ID No. 20

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TTAAAGGCCCGA
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SEQ ID No. 21

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CGGAGCGCCATTGTCTCGAATCGAAATAGCACACCTAGCATATGCAAGAC
GAAATTATACAAAATGTATTGATCAACTTAATAAATGCTTTGCTAAAGAA
CTTGACAATCTACCTGCAGAGAGTTTAGAGGAATATGCTCTTTCCTTACT
TAAAGGAAATAACACTAAGGAATTTGAAAAATTAGTAGAAAGTTCTCTAA
GTGAGAAGTTTAAAGATCGAACTTTCTTTAAAAAAGAATATGTTTCCTTT
CTTGCTAAAAATCAGTTATGGAAAAAATTAGTTAAATATGCTAGCAATTG
GGCATTACAAGATAAAGAAAAATTCAATTATCCATTAATGCTTGCTCATT
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GAAGAACATCCCTATGAAT
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SEQ ID No. 22

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TAAATTGACGGAAGGAA
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SEQ ID No. 23

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AATTATTTAAAGGCGTCTATCATATTGCAAAAGACGAAACCTATCTTTAT
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TACGTGAAGAATTAGAACTTGTACAAGGTGCATCAAACGAATTTGATCAC
GAAGCCTTTATCAACGGCGAACTCACACCGGTATTCTTCGGTACCGCATT
AGGTAACTTCGGTGTGGATCATTTCCTAGACGGTTTAACCGAATGGGCGC
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GAAAAATTCTCTGGCTTCGTGCTTcCTgTTAAGATcCAAGCGAATaTGGG
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ACcGAAAcAcGGTCTTCAAACTCAAACACCTACGTATCGcCAtAAGATGT
CTGCGATTTCCAGATGCCTTAACCTTTATGGcGGGTGAACGTGCACATGC
TcGAAGAAGCTTACgCGCgGCctATATTATCGGTTTACATAACCACGGAA
CAATCCAAtTcGGtGACACCTTCAcTCAcGGTGAAGTGATGAAATTTACC
GccTTCC
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SEQ ID No. 24

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CGGCTTACCGGTTGAAATCCAAACCACCGAATATGATGAAGAAGGCAAAG
CAAGCGGTCAAAAATGGGAAAAAATTAACAAAGCGCAAGCACTTTGGACT
CGTTCAAAAACCGAAATTTCAGACGAGGAATACCAAGAGTTTTATAAACA
TTTAAGCCACGATTATAATGACTCGCTCATTTGGGCGCATAATAAAGTAG
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CG
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SEQ ID No. 25

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AACTTGTTGTTTATTCGCAAAATCTTCACCAACGCCTTCTTTTAACACTAAACCGAATTCGTTCC
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GTTAATGCCGCACGTAGTGAAGCAGTAACTTTATTCTCTTGTAAAATCTCACGTGAAACGTTGAG
CGGCAAGTCATTCGTGTCTAATAAACCCCGCATAAAACGTAGGTAATTCGGCATAAACACTTTCG
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SEQ ID No. 26

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cACTTAGCCCCCAAATTAGCAGTTCCTAAAAAAAAGCCTCCCTCAAAGGAAGTAAAAAAAGCGCC
TGAAAAGATCATGAAaAAACATGAGACAAAGAAAGAtGTTTCTCCAACAAACAAAAGCAGTCGTA
CTCAGCAGCCGATCCAACAAGGAAATCCAAATGGTATAAATGGAAGCAAATCAACCTATGGAAAT
GCCAAAGTCGATGCCTCCCTTGGTGCTGGTTATGGCAATGCAATGCGAGGGCCGTTGCTCTGATA
TTTCAGATGAATCTGATGATG
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SEQ ID No. 27

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TGTCGCAATGCAGATTTAGCTTGTGAAGTGACGTTACAGCCGCTACGCCC
GCTACGCCGCTATGCATTGGATGCGGCGATTTTATTCTCCGATATTTTAA
CCGTTCCGGATGCAATGGGCTTAGGTTTAAGTTTCGGCGCGGGTGAAGGG
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GATTCCGGATCCGGAAAGCGAATTACAATATGTGATGAATGCGGTACGTA
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AGCTCGTGGACATTGGCGACTTATATGGTGGAAGGCGGCTCAAGCAAAGC
GTTCACCAAAATTAAAAAAATGATGTATGCCGATCCGAAAATTTTACACG
CATTATTAGATAAATTAGCCGATAGCGTGATCTTATATCTGAATGCACAA
ATTAAAGCCGGCGCGCAAGCGGTGATGGTGTTCGATACTTGGGGCGGCGT
GTTGGCGCATAACGAATATAAAGAGTTCTCGCTACGTTATATGCACAAAA
TCGTggacggtttaattcgtgaaaacgaaggcaga

SEQ ID No. 28

TGCGTGGTATCCaCACGATGGAACACTTATTTGCCGGCTTATGCCGTGAT
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TGGTTTCGGCGTGGACAAAATCGATGGAAGATGCTTTAAATAAAGTACCG
GACGTGTCAAAAATTCCGGAATTAAACGAATATCAATGCGGCTCTTATAA
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AGGGTATCGGCGTAAACCGTAACGAAGATTTAGCGCTTGATGAGAAATTA
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TCTTAAAAATATATTTTAATCTTTGACAGACGCTTATGGCGTcTTTTAGT
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SEQ ID No. 29

CGCAGTGTAAGTTTTTGCCCGATTGCACGATGTAGAAGAAGTGCGGTTACTGATGAATCCACGCC
GCCTGATAAACCTAAAATGACTTCGTCATCGCCTACTTG

SEQ ID No. 30

TAGAAAAACTCTGAAGTCCTTGAGAATTTCCGCTCTTCGCCATAGCCTGT
AACGCTTGGGTCGGCACGTGTAATAAGCTGTTGGTCAGTTTATAACTTAG
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ACGATATCCGCTTGGTTTAAACCGATTTGCAATGCGCTTAATGATAAAAT
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ACCATTCAAAACATTGTTCACGCCATGCGATGTTATCTTCATGTTCTTCT
CGCGGCGTAATTTCCGCATTATGAAAATAAAGCTCTGTTCGGTTACACGT
TGAAAGAATGACAACGCTTTCAGCAAGTGCGCGGGGGTTGAATTTGCTCA
A

SEQ ID No. 31

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TACATATCAACAAGGATACTTTGAATACGAGAAAGATGGATTTGGTCCTGTAGCTGAAACATTAG
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SEQ ID No. 32

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SEQ ID No. 33

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AATAATTCTTCCACACGAGGCCAGTTGTTCGGGCGGCTTACGTCCATTGCGTTTGAAAGCGTTGC
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TTGATGCGATAAAACGTTTAATTGGTAAACCTAAGGTTTTTGCGATTAAACCAGCGGTTAAGTTA
CCGAAGTTACCGCTTGGTACTGAAACCACTACATCAGAACGTTTTGCTTTCGGTAATTGTGCTAC
CGCTTCAAAGTAATAACAAACTTGCGCTAATAAACGGCTGATATTGATTGAGTTTTGCGGAGTTT
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SEQ ID No. 34

ATTGTTTCCGATATCCTTGAGTTAGTAAATCCTGATTTAGCCCATATTCA
TTGTATTCAAGGCTTAGGCGTTGGTGTTGTTGATGCATGCACACAACGAA
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CAATTTATGATTAAAGCTGATGGTAAGTTCTGTCATCAATATAAAATTGA
TAAAAATGAATGTATAAATGTATTGGTAGTACATCAGAAT

SEQ ID No. 35

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GCCCCTACGGGATCAACAAAGTGGATATGCGTACCGACTAAACCGGCAGGATGAACACCGCTAAA
CGACTTAATTGTGATACCTTCAATCGCCGGACTTAACGGGATATTGCTGTCCGCGTCTTTACATA
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SEQ ID No. 36

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GGTAATACGATATTTTCTACTTTCTTAAGTGTAATTGCGAATTTCGCCGCTTTACCTTTTAAGTT
TTCAGCGTGGTATTcTTCAGGGAAAGTAACATCGATATCGAATTGtTCGCCGGCTTTGTGACCAA
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SEQ ID No. 37

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CGGTTGAAGTTGTACTTGCGAAAGCAAAAGTAACTGAAAAAGCGACTTCT
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GCTCTCCGGTTTGGAGGGCTTTTAATTAGCATTTTATAAGCGGTTGATTT
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SEQ ID No. 38

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AATTGTGTGCGACTTTATTTAAACATATATTTCCTGAGGTGATGGCATAT
GCCAGTAATTAAAGTTCGTGAAAATGAATCATTTGACGTTGCATTACGTC
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CGTGAATTCTATGAAAAACCAACTACAATTCGTAAACGTGAAAAAGCATC
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ACCG

SEQ ID No. 39

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SEQ ID No. 40

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GCAAT

SEQ ID No. 41

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SEQ ID No. 42

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SEQ ID No. 43

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SEQ ID No. 44

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ATTCACACGTACGTGATGCGTTAGATTCTAAACGTATGATGATTATCGTATGGTTAGCGTTATTC
CCGGCa
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SEQ ID No. 45

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ATATTATTTGGACTATATAATTGGATTGCTAATGCAATTAATAATATAGA
AACACCAACAGGAACCATTATGATTTCTGCATTGTTTATTTTAGTAGGAA
TTCATTTATTACTCTCTTTTATATCTTATGATGTAAATAATTATCCAACG
GAATCAATTAGTAAAAATTTAGATGAATAATCCCATTTGTTCTGTTATTG
TTTGTTATGAACCAACTAAGAAAATATTTTTTTTAATCAACTCTCTAATT
AAGCAGAATGTATAT
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SEQ ID No. 46

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CTTGTCTGTTTTTTAGTCAGATAAT
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SEQ ID No. 47

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GCTTTAGCAGCTTAATAACCTGCTCATAGCCTTATCGCCTCAGCTTCCGC
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SEQ ID No. 48

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SEQ ID No. 49

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SEQ ID No. 50

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SEQ ID No. 51

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SEQ ID No. 52

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TCGAAAGAGAGTATTTCGGAGTTAGGATTAAAAACAGCATTTATCTCTAA
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SEQ ID No. 53

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TCTTTAATCATGTtGAAGATaCTGTtGA
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SEQ ID No. 54

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CTATCAATGGGTGtGTGAAGGCAAAATCGATAaTGTTATCgCAGTGGTCGGTTTACAATGGTTAC
AACTTAATTATCAAAAATATCGAGTGTAATGAGCGTTTTCCGATAACGACGAATaTATTTTGCCC
TCTTTAATGAGGGCAAATTTTTTGaagtat

SEQ ID No. 55

CGCTCATCACCAAACACTTGAGGTTCTAAGAGTTTTACATCCGGAAT

SEQ ID No. 56

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GAATTAATCAAAAGCCCCATAGTTTATGCTTTGGGGCTTTAATTTTTTCATCAGTTTTTAAATTA
AGCGGTCAAAAATTAAGAATGTTTTGCAAATGAAATTAGTTGAAAT

| | | | Similarity to: | | | | | |
Class	Strain	Gene		%	Span	Number	Known or Putative Function:	In vitro CI:	In vivo CI:
Cell Surface	9C2	cpxC				AAD30161	capsular polysaccharide export inner membrane	1.020	3.88E-03
	26A9		lcbB (Nm)	37(50)	82	AAF21951	LPS or capsule biosynthesis	1.068	
	9B7	galU				AAC28326	LPS core biosynthesis	1.750	
	25B7	rfbU				AAG45944	LPS O-antigen biosynthesis	0.0014	
	10B11	rmlC	rfbC (Aa)	100	15	AAG49406	LPS O-antigen biosynthesis	0.232	
	12D5	rfbN				AAG45942	LPS O-antigen biosynthesis	0.024	
	21B8	rfbP				AAG45943	LPS O-antigen biosynthesis	0.029	
	15A9	hypothetical glycosyltransferase	PF0798 (Pf)	37(55)	270	gi:18977170	LPS O-antigen biosynthesis	0.739	
	4D4		lppB (Hs)	46(63)	83	AAK03698	lipoprotein B precursor	1.870	
	17A4		ompP2 (HI)	40(55)	237	Q48221	Outer membrane protein P2 precursor	1.149	
Metabolism	10B12		adpP (HI)	61(76)	62	P44684	ADP-ribose pyrophosphatase	1.029	
	10A11		argG (HI)	65(88)	34	AAC23373	argininosuccinate synthase	0.802	
	33C7		atpA (HI)	89(95)	134	P43714	ATP synthase	0.314	
	17B8		ccmH (HI)	53(77)	166	P46458	cytochrome C biogenesis protein	1.100	

Clone	Gene	Gene (organism)	Identity	Length	Accession	Description	Value 1	Value 2
35D11		dsbA (HI)	63(77)	160	P31810	thiol:disulphide interchange protein precursor	1.040	3.43E-04
26C3		hemA (Pm)	61(74)	394	CAA71452	Glutamyl-tRNA reductase	0.048	
4B9		moaA (HI)	67(74)	114	P45311	molybdenum cofactor biosynthesis protein	1.150	
19D5	mrp				AAD01696	ATPase	0.200	
29B11		napB (Pm)	55(70)	77	AAK03681	periplasmic nitrate reductase	1.050	1.093
9B5		pnp (Pm)	75(79)	125	AAF68421	polynucleotide phosphorylase	1.180	
0A7		pntB (HI)	68(77)	458	P43010	NAD(P) transhydrogenase	0.785	
23C9	aopA/nqrA				AAC43631	Na-translocating NADH-ubiquinone oxidoreductase	0.716	
29A10		prfC (HI)	73(81)	342	P43928	peptide chain release factor	4.670	
29B12		recR (HI)	81(87)	71	P44712	Recombination protein	3.600	
26A10		thrC (HI)	88(93)	190	P44503	Threonine synthase	0.867	1.598
0F6		tonB (Hd)	64(74)	288	O51810	Energy transducer	2.488	2.20E-02
27A12	tonB				Y17916	Energy transducer	0.902	1.54
26D5		uroD/hemE (Pm)	88(93)	230	AAK03818	Uroporphrinogen decarboxylase	NA*	
15A11		visC (Pm)	55(82)	43	AAK03810	monooxygenase	0.684	
26A6		yjbK (HI)	70(84)	127	P44868	probable tRNA/rRNA methyltransferase	0.599	
33B7		yjfH (HI)	77(85)	157	P44906	probable rRNA methylase	0.744	

Category	Clone	Gene	% identity	aa	Accession	Description	Value	p-value
Regulatory	26D12	guaA (Hi)	83(85)	43	P44335	GMP synthase	1.586	
	6C12	fur (Hd)	79(83)	139	P71333	ferric uptake regulator	0.627	2.76E-02
	26D3	luxS (Hi)	69(84)	118	P44007	autoinducer-2 production protein	2.063	
	19B10	mlcA (Pm)	45(69)	61	AAK03872	negative regulator of rpoE	1.725	6.50E-02
Stress	21D3	rpoE (Hi)	81(90)	189	P44790	Sigma factor E	0.789	0.88
	13A3	dnaJ (Hd)	85(99)	14	P48208	chaperone protein	0.163	
	26B6	htpG (Ec)	69(82)	368	P10413	Heatshock protein	1.251	
	13D8	lon (Pm)	74(86)	292	AAK04062	ATP-dependent protease	0.674	
	13C1	prc (Pm)	65(79)	290	AAK02353	tail specific protease	1.324	
	22A10	tig (Pm)	50(61)	152	AAK04059	Trigger factor involved in cell division	0.851	
	9D10	corC (Hi)	66(78)	248	Q57368	Magnesium and cobalt efflux protein	3.380	
Transport	19D1	Hypothetical ABC (Pm)	79(89)	110	AAK03812	ABC transporter	1.341	
	0C5	mglA (Pm)	81(86)	242	P44884	galactoside ATP transporter	0.651	1.80E-02
	0D6	Hypothetical membrane protein (Pm)	56(76)	210	AAK03081	Probable membrane protein	1.268	
	32A7	Hypothetical ABC (Pm)	60(73)	370	AAK03080	Probable ABC transporter	0.962	
	13B12	yfeB (Pm)	75(86)	185	AAK02483	Iron (III) ATP-binding protein	0.922	
	35D1	znuA (Hd)	71(78)	176	AAF00116	periplasmic zinc ABC transporter	0.450	

					Unknown		
14D5	No homology A				Unknown	0.953	5.34E-03
2D5	No homology B				Unknown	1.245	1.03E-02
32A11	Unknown (Pm)	31(52)	124	AAK03268	Unknown	2.950	0.991
4C1	Unknown (Pm)	42(59)	327	AAK02354	Unknown	1.110	0.935
9A4	Hypothetical protein (HI)	53(74)	91	P44027	C4-decarboxylate transport protein homologue	1.018	